PHOTOBIONT DIVERSITY IN TELOSCHISTACEAE (LECANOROMYCETES)

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Zusammenfassung

Die Teloschistaceae sind eine Familie Flechten bildender Ascomyceten. Sie umfassen, nebst vielen Endemiten mit kleinem Verbreitungsareal, einige sehr häufige und weit verbreitete Arten. Eine davon, die Gelbe Wandflechte (*Xanthoria parietina*), ist der bestuntersuchte Flechtenpilz überhaupt. Teloschistaceae leben in Symbiose mit einzelligen Grünalgen der Gattung *Trebouxia* (Trebouxiophyceae), jedoch ist das Spektrum compatibler Algenarten pro Pilzart von keinem Vertreter dieser Familie bekannt. Wie die meisten Flechten bildenden Pilze sind die Teloschistaceae physiologisch fakultativ biotroph, d.h. sie können isoliert und sterilkultiviert werden; das Gleiche gilt für ihre Algenpartner. Leider wird der aus der Natur bekannte symbiotische Phänotyp in Resynthese-Experimenten unter Laborbedingungen nicht exprimiert. Deshalb muss das Spektrum akzeptabler Algenarten pro Pilzart an im Feld gesammelten Flechtenproben ermittelt werden.

In der vorliegenden Dissertation wurden die Photobionten von 12 *Xanthoria*-Arten (darunter 3 südafrikanische Endemiten), 7 *Xanthomendoza*- und 2 *Teloschistes*-Arten sowie von *Josefpoeltia boliviensis* mit molekularen Markern (ITS 1&2, 5.8S rDNA und plastidäre *rbc*L-Region) untersucht. Sämtliche der hier untersuchten Flechtenpilz-Arten bilden morphologisch komplexe Thalli mit innerer Schichtung; die Algenzellen sind in der sog. Algenschicht lokalisiert. Von den meisten Arten standen mehrere Belege zur Verfügung; die grösste Anzahl Proben (63) wurde von *Xanthoria parietina* aus 4 Kontinenten bearbeitet. Pro Algenart wurden mehrere Genotypen identifiziert. Sämtliche *Xanthoria*-Arten waren mit nur zwei *Trebouxia*-Arten assoziiert (*T. arboricola*, *T. decolorans*), welche zur „*arboricola*"-Gruppe gehören. Fünf der sieben *Xanthomendoza*-Arten waren mit den gleichen Algenpartnern aus der „*arboricola*"-Gruppe vergesellschaftet, während die Thalli der beiden anderen entweder *T. impressa* oder *T. gelatinosa* beherbergten (beide aus der „*impressa*"-Gruppe).
Neue „group 1" Introns wurden in der LSU und SSU rDNA von *Trebouxia*- und *Asterochloris*-Arten entdeckt. Beim Vergleich der Phylogenie dieser Introns mit der ITS-Phylogenie wurden einige interessante Fälle entdeckt, die auf lateralen Intron-Transfer hinweisen.

Die intraspezifische genetische Diversität von *Trebouxia arboricola*- und *T. decolorans*-Photobionten in Populationen von *Xanthoria parietina* wurden an sterilkultivierten Isolaten mittels „fingerprinting"-Methoden untersucht (RAPD-PCR; randomly amplified, polymorphic DNA als „multilocus approach"). Epiphytische und saxicole Proben wurden systematisch gesammelt in Populationen an der Meeresküste (Bretagne), in ländlichen (SW-Frankreich, Burgund) und städtischen Gebieten (SW-Frankreich, Irchel-Park Zürich). Da sich *Trebouxia*-Arten nicht sexuell vermehren und überdies laut Literatur ausserhalb von Flechten-Thalli selten freilebend vorkommen sollen, stellt sich die Frage, ob die innerhalb einer *Xanthoria*-Population vorkommenden Trebouxien kolnal oder genetisch verschieden sind. In allen Populationen wurde eine unerwartet hohe genetische Diversität festgestellt. Da sexuelle Reproduktion und Rekombination fehlen, kommen Mutationen und/oder Durchmischung in Frage. Dabei dürften Wirbellose und Wirbeltiere als Vektoren für den Kurz- und Langstreckentransport in Frage kommen.

In einem kleinen Nebenprojekt wurden die Photobionten von *Psoroglaena epiphylla* und *P. stigonemoides* (Verrucariales) identifiziert, welche für Lichenologen und Phycologen von Interesse sind. Laut Literatur sollen die lebhaft grünen Photobionten in den mikrofilamentösen Thalli dieser Flechtenpilze zu den Prochlorophyten gehören, jenen bisher nur marin bekannten Cyanobakterien mit Chlorophyll a & b, aber ohne Phycobiline. Dies wären die ersten je entdeckten terrestrischen Prochlorophyten gewesen. Mit ultrastrukturellen und molekularen Markern (ITS und rbcL-Region) wurden die Photobionten beider Flechtenpilze als einzellige Grünalgen aus den Trebouxiophyceae identifiziert.

Summary

The Teloschistaceae are a family of lichen-forming ascomycetes comprising some very common and widespread species. One of them, the yellow wall lichen (*Xanthoria parietina*), is the best investigated lichen-forming fungus. Teloschistaceae are symbiotic with unicellular green algae of the genus *Trebouxia* (Trebouxiophyceae), but the range of acceptable algal species per fungal partner was so far unknown. As physiologically facultatively symbiotic fungi Teloschistaceae, like the majority of lichen-forming ascomycetes, can be isolated into sterile culture; the same applies for their green algal partner. As the symbiotic phenotype, as seen in nature, is not expressed in resynthesis experiments carried out under laboratory conditions, the range of acceptable photobiont taxa per fungal species has to be explored in samples collected in the field.

In this thesis the photobionts of 12 *Xanthoria* species (including 3 South African endemic species), 7 *Xanthomendoza* spp., 2 *Teloschistes* spp. and *Josefpoeltia boliviensis* were investigated with molecular markers (ITS 1 & 2, 5.8S rDNA and *rbc*L region). All of these fungal species form morphologically complex thalli with internal stratification. From most taxa several samples from different geographic locations were available, the largest sample size being 63 specimens of *Xanthoria parietina* from worldwide locations. Several genotypes per algal species were identified. All *Xanthoria* species were associated with only 2 *Trebouxia* species (*T. arboricola*, *T. decolorans*) out of the "*arboricola*" clade, indicating considerable selectivity of the fungal partners towards their photobiont. Five out of seven *Xanthomendoza* species associated with the same *Trebouxia* spp. from the "*arboricola*" clade as the *Xanthoria* spp., the other two had either *Trebouxia impressa* or *T. gelatinosa*, both belonging to the "*impressa*" clade.

Novel group I introns were found in the LSU and SSU rRNA of *Trebouxia* and *Asterochloris* species. Intron phylogenies were compared with ITS phylogenies of a wide range of *Trebouxia* species. Several arrangements were interpreted as being, with high probability, the result of lateral transfers.

The intraspecific genetic diversity of *Trebouxia arboricola* and *T. decolorans* photobionts in populations of *Xanthoria parietina* was analyzed with fingerprinting techniques applied to sterile-cultured isolates, using RAPD-PCR (randomly amplified polymorphic DNA) as a multilocus approach. Epiphytic and saxicolous samples of *Xanthoria parietina* were systematically collected in coastal, rural and urban sites in NW, SW and central France and on the University campus (Irchel-Park) in Zürich, Switzerland. As *Trebouxia* spp. do not sexually reproduce and, moreover, are assumed to be rare outside lichen thalli, the question was whether the *Trebouxia* cells contained in thalli of local populations of *Xanthoria parietina* are clonal or genetically diverse. In all populations a surprisingly wide spectrum of algal genotypes was detected, which, in the presumed absence of sexual reproduction and recombination, might result from mutations and/or from mixing due to short and long distance transport via vertebrate and invertebrate vectors.

In a small side project the photobionts of *Psoroglaena epiphylla* and *P. stigonemoides* (Verrucariales) were identified, which are of general interest among lichenologists and phycologists. In the literature the vivid green photobionts contained in the microfilamentous thalli of representatives of the genus *Psoroglaena* were hypothesized to belong to the prochlorophytes, a group of marine cyanobacteria containing chlorophyll a and b, but no phycobilins. This would have been the first terrestrial prochlorophytes ever found. With ultrastructural techniques and molecular markers (ITS and *rbc*L regions) the photobionts of both *Psoroglaena* species were identified as green algae belonging to the Trebouxiophyceae.

1 Introduction

1.1 Lichen symbiosis

1.1.1 Lichen-forming fungi and their photobionts

Lichens are the symbiotic phenotype of nutritionally specialized, ecologically obligate biotrophic fungi which acquire fixed carbon from a population of minute photobiont cells (Honegger, 1996). Species names of lichens refer to the fungal partner; lichen photobionts have their own names and phylogenies (Hawksworth, 1988). Approximately 13’500 species of lichen-forming fungi were so far described; thus every fifth fungal species is a lichen (Hawksworth, 1988). Approximately 98% of lichen-forming fungi are ascomycetes, less than 1% are basidomycetes and the rest are conidial fungi or taxa with no vegetative or sexually produced spores; these disperse via thallus fragmentation (summary: Honegger, 1996). The majority of lichen-forming fungi are terrestrial, only few freshwater and marine species have been reported.

About 100 species of lichen photobionts were so far described; thus many species are partners of a wide range lichen-forming fungi (referred to as mycobionts). About 85% of lichen photobionts are green algae, about 10% are cyanobacteria. 3-4% of lichen-forming fungi associate simultaneously with both, a green algal and a cyanobacterial photobiont (Tschermak-Woess, 1988). Among the few marine lichen-forming ascomycetes a small number of species associate with Xanthophyceae or Phaeophyceae (Tschermak-Woess, 1988; Sanders et al., 2004). Lichen photobionts are less intensely studied than their fungal partners. In less than 2% of lichens has the photobiont ever been identified at species level; fairly often not even the generic affiliation is known (see chapter 5).

First fossil records of lichen-like associations were reported from a marine habitat in the approximately 600 million years old Doushantuo Formation in South Western China (Yuan et al., 2004). Because of their intimate association lichen symbionts were hypothesized to have co-evolved. As fossil records are so far

very scarce, phylogenetic analyses of both partners might give insights in coevolutionary traits.

1.1.2 Specificity and selectivity in lichen symbiosis

Although lichen-forming fungi are almost exclusively found in the symbiotic state in natural habitats, most of them can be isolated into sterile culture, where they grow in the aposymbiotic state. Many lichen photobiont species are known to occur also in the free-living state, but others are mostly confined to the thalli of lichen-forming fungi, yet all of them can be axenically cultured (reviews: Tschermak-Woess, 1988; Honegger, 1992). Generations of lichenologists tried to re-synthesize lichens under laboratory conditions by combining sterile cultured isolates of both symbionts. Although lichen-forming fungi contact and overgrow their photoautotrophic partners in sterile culture, they express their characteristic symbiotic phenotype, as known from nature, only very rarely and unpredictably under laboratory conditions. As ultimately non-compatible photobiont cells are also overgrown and partly parasitized by cultured mycobionts (Ahmadjian & Jacobs, 1981), the range of compatible partners can not be explored in such resynthesis experiments (review: Honegger, 1993). So far no standardized protocols for successful resynthesis experiments are available. Thus the range of compatible combinations was explored in material collected in the field (methods see below).The majority of lichen-forming fungi seem to be moderately specific to specific, but highly selective as regards to photobiont acquisition. Specificity was defined as the range and taxonomic relatedness of acceptable partners (Smith & Douglas, 1987). In a moderately specific association several closely related photobionts are acceptable partners, in a specific one only one species is accepted (review: Honegger, 1996). Selectivity was defined as the preferential interaction of the organisms (Galun & Bubrick, 1984). The degree of selectivity varies depending on the taxa studied and the taxonomic level of comparison. Highly selective fungal partners always associate with the same algal species, even when other algae are distinctly more common in the same habitat. The algal partner is not randomly selected by the fungal partner (Friedl, 1989b; Beck et al., 1998; Rambold et al., 1998; Kroken & Taylor, 2000; Dahlkild et al., 2001; Piercey-Normore, 2004). The majority of morphologically advanced species of lichen- forming fungi

associate with one or few species of green algae or cyanobacteria, but *Umbilicaria* spp. from Antarctica (Romeike et al., 2002) and many taxa with crustose thalli worldwide show lower specificity towards their photobionts (Friedl, 1987; Tschermak-Woess, 1988; Helms, 2003). Lichen mycobionts are more selective than the photobionts; therefore no co-speciation (congruent phylogenies of the two bionts) was detected (Beck et al., 2002).

Many families of lichen-forming ascomycetes have affinities for certain clades or guilds of photobionts. Examples are the Peltigeraceae, which are all symbiotic with cyanobacterial photobionts of the genus *Nostoc*, some species forming triple symbioses with a green algal partner of the genus *Coccomyxa*, or the Parmeliaceae, comprising more than 2100 species, which are all symbiotic with species of the green algal genus *Trebouxia* comprising less than 20 species (see below). Contrarily in some families or even genera of lichen-forming ascomycetes a wide range of photobionts was found, but individual fungal species in these groups are moderately specific to specific, i.e. associate with one or few closely related photobiont taxa. Examples are the Verrucariaceae with photobionts belonging to diverse green algal genera, one xanthophyceaen and one phaeophycean genus (Tschermak-Woess, 1988; Sanders et al., 2004), or the genus *Chaenotheca* (Caliciales), comprising approximately 20 species worldwide, which associate with photobionts belonging to 4 green algal genera (*Dictyochloropsis*, *Stichococcus*, *Trebouxia*, *Trentepohlia*) belonging to 2 classes (Trebouxiophyceae, Ulvophyceae; Tibell, 2001).

1.2 Green algal lichen photobionts in focus

1.2.1 Green algal taxonomy

With the advent of new technologies in the last 40 years algal taxonomy in general and green algal taxonomy in particular underwent thorough changes. The massive gain in resolution in electron microscopy, as compared to light microscopy, allowed visualization of ultrastructural details such as plastid membranes, pyrenoids, elements of the cytoskeleton including the spindle apparatus, flagellae and their basal body, microtubule organizing centers and details of cy-

tokinesis, all of which are below the resolution limits of the light microscope. Biochemical features such as photosynthetic, accessory and other pigments of plastids or enzymes involved in biochemical processes became important elements in new taxonomic concepts. Molecular tools brought revolutionary changes in the classification of all living organisms. At present four monophyletic classes of green algae are recognized, which fall in two major evolutionary clades: Ulvophyceae, Trebouxiophyceae and Chlorophyceae (the so-called UTC clades) in the first and Charophyceae in the second, the latter revealing monophyly with land plants (Mishler et al., 1994; Friedl, 1995; Graham & Wilcox, 2000; Lewis & McCourt, 2004). Table 1.1 shows three genera of unicellular green algae, which contain photobionts of lichen-forming fungi, and the changes in their classification within the last 30 years.

Table 1.1 Classification of three genera of green algae comprising lichen photobionts, as seen in the recent literature

Authors	Green algal genera		
	Trebouxia de Puymaly	***Chlorella*** Printz	***Stichococcus*** Nägeli
Light microscopy			
Archibald, 1975†	Chlorophyceae, Chlorococcales, Chlorosarcinales		
Bold & Wynne, 1978	Chlorophyceae, Chlorococcales	Chlorophyceae, Chlorellales	Chlorophyceae, Ulotrichales
Tschermak-Woess, 1988	Chlorophyceae, Chlorococcales,	Chlorophyceae, Chlorococcales,	Chlorophyceae, Klebsormidiales,
Electron microscopy			
Mattox & Stewart, 1984	Pleurastrophyceae, Pleurastrales	Chlorophyceae, Chlorococcales?	Charophyceae, Klebsormidiales
Melkonian, 1990	Chlorophyceae, Microthamniales	Chlorophyceae, Chlorococcales?	
Molecular markers			
Friedl, 1995; 1996; 1997; Friedl & Rokitta, 1997	Trebouxiophyceae, Trebouxiales	Trebouxiophyceae, Chlorellales‡	
Graham & Wilcox, 2000	Trebouxiophyceae, Microthamniales	Trebouxiophyceae, Chlorellales	
Lewis & McCourt, 2004	Trebouxiophyceae, Trebouxiales	Trebouxiophyceae, Chlorellales	Trebouxiophyceae, Prasiolales
Karsten et al., 2005	Trebouxiophyceae, Trebouxiales	Trebouxiophyceae, Chlorellales & *incertae sedis*	Trebouxiophyceae, Prasiolales

† genus *Trebouxia* was placed in Chlorococcales and the newly described genus *Pseudotrebouxia* placed in Chlorosarcinales. *Pseudotrebouxia* is not accepted in modern concepts. ‡ *Chlorella ellipsoidea* and related species are placed under Chlorellales within Trebouxiophyceae, while other, morphologically very similar *Chlorella* spp. are *incertae sedis* within Chlorophyceae.

1.2.2 Trebouxiophyceae and the “lichen algae”

Based on ultrastructural data, the free-living, filamentous *Pleurastrum insigne* with its metacentric spindle was found to differ considerably from Chlorococcales (Molnar et al., 1975). The same features were detected in *Trebouxia* and *Friedmannia*. Thus a new class Pleurastrophyceae with one order, Pleurastrales, was erected (Mattox & Stewart, 1984). Melkonian (1990), also focusing on

ultrastructural data such as the semiclosed mitosis, and cytokinesis by means of a cleavage furrow guided by the microtubules of the phycoplast, transferred *Trebouxia* and *Microthamnium* to a new order Microthamniales within the Chlorophyceae. Based on phylogenetic analyses of 18S ribosomal DNA sequences Friedl (1995) erected the new class Trebouxiophyceae within the Chlorophyta.

With increasing numbers of sequences available the Trebouxiophyceae turned out to comprise a large number of genera and species which had been formerly classified in different classes and orders of the green algae (examples in Tab. 1.1). At present Trebouxiophyceae include four orders: Chlorellales, Microthamniales, Prasiolales, and Trebouxiales and a group *incertae sedis* (Lewis & McCourt, 2004; Karsten et al., 2005). The vast majority of lichen photobionts belong to Trebouxiales (genera *Trebouxia*, *Asterochloris, Coccomyxa, Dictyochloropsis, Dilabifilum, Elliptochloris, Myrmecia, Stichococcus* etc.), few to the Chlorellales (*Chlorella*) or Prasiolales (*Prasiola*). Thus Trebouxiales are often referred to as “lichen algae”. Beside lichen photobionts Trebouxiophyceae also comprise unicellular green algal photobionts of protists and invertebrates (*Chlorella* spp.) and numerous genera of free-living algae. The other group of important and widespread green algal photobionts of lichen-forming fungi belongs to the Trentepohliales within the Ulvophyceae (genera *Trentepohlia*, *Phycopeltis* and *Cephaleuros*, especially in subtropical and tropical regions).

1.2.3 The genus *Trebouxia*: most common and widespread lichen photobionts

The genus *Trebouxia* de Puymaly (1924) comprises approximately 25 species of unicellular green algae, which are estimated to be the photobionts of >50% of all lichen-forming fungi worldwide. Distinctive feature at the light microscopy level is one large, centrally located chloroplast per cell with central pyrenoid, the nucleus and all other cell organelles being located at the cell periphery. Type species of the genus is *T. arboricola*, formerly described as *Cystococcus humicola* Nägeli (1849).*Trebouxia* species were described on the basis of morphological criteria, mainly on cell shape (globose or ovoid) and lobation of the chloroplast (Ahmadjian, 1960, 1967; Ettl & Gärtner, 1984; Gärtner, 1985a; Friedl, 1989a).

The genus *Trebouxia* has seen many revsions based on light microscopic investigations. Already Ahmadjian (1960) realized that zoospores and aplanospores are formed by all, vegetative cell division only by some species of the genus *Trebouxia*. Thus he distinguished group I without, and group II with vegetative cell divison. Archibald (1975) reported on different modes of cell division, which seemed to justify splitting of the genus in *Trebouxia* de Puymaly, comprising species with “eleutheroschisis” (no vegetative cell division, autospores missing), and *Pseudotrebouxia* Archibald, comprising species with “desmoschisis” (vegetative cell division, with autospores); she classified the genus *Trebouxia* in Chlorococcales, *Pseudotrebouxia* in Chlorosarcinales within the Chlorophyceae. Although this revision was not accepted by leading experts (e.g. Gärtner, 1985b; Tschermak-Woess, 1989) and the genus *Pseudotrebouxia* is not retained in modern concepts, the genus name *Pseudotrebouxia* is still found in the recent literature.

Tschermak-Woess (1989) proposed division of the genus *Trebouxia* in 2 subgenera, *Trebouxia* s. stricto comprising autospore forming species, subgenus *Eleutherococcus* comprising species without autospores. As she had described the photobiont of the lichen-forming ascomycete *Varicellaria carneonivea* as *Asterochloris phycobiontica* gen. et spec. nov. (Tschermak-Woess, 1980), she pointed out that this taxon has to be transferred to the subgenus *Eleutherococcus* as *Trebouxia phycobiontica* and, should this subgenus ever be given the rank of a genus, *Asterochloris* would be the valid name.

1.2.4 *Trebouxia* s.str. and *Asterochloris* (Tscherm.-Woess) Friedl *ined*.

Based on his detailed comparative transmission electron microscopy studies, focusing on cell cycle, cell shape, chloroplast shape, arrangement of thylakoids and fine structure of the pyrenoid, Friedl (1989) distinguished 8 natural groups within the genus *Trebouxia,* each with 1 to 5 morphospecies. Six out of these 8 groups comprise species with autospore formation. He proposed to set some of the species names in synonymy, thus reducing the number of species from 26 (Gärtner, 1985a, b; Friedl & Gärtner, 1988) to 16. In his outline of the genus *Trebouxia* Takeshita (2001) added no new insights, but proposed to maintain

17 species.

Molecular data sets, focusing on the phylogeny of nuclear ribosomal small subunit (nrSSU; Friedl, 1995), large subunit (nrLSU; Friedl & Rokitta, 1997) and the internal transcribed spacer region (Tab. 1.3) indicated paraphyly of the genus *Trebouxia* s. l. In a publication by Rambold et al., (1998) Friedl announced the new genus *Asterochloris* (Tschermak-Woess) Friedl, which, unfortunately, never has been validly published. Thus it s referred to as *Asterochloris* (Tscherm.-Woess) Friedl *ined*.

It is interesting to see that ultrastructural and molecular data sets on representatives of the genera *Trebouxia* and *Asterochloris* are largely, but not fully congruent (see Table 2.4). Species names given to representatives of the genera *Trebouxia* and *Asterochloris* are based on morphological characters achieved with light microscopy techniques. Before the advent of molecular techniques identification of lichen photobionts at species level necessitated isolation into sterile culture and comparison with sterile cultured isolates of the type species, a time-consuming work requiring much expertise. Culturing under standardized conditions was required because of morphological changes of the photobionts in the symbiotic state within lichen thalli. Due to the ease of molecular investigations, which can be carried out with DNA extracts of whole lichens, morphological investigations are largely avoided. In phylogenetic analyses of sequence data of representatives of the genus *Trebouxia* s. str. 4 clades became evident, each being named after a representative morphospecies: “A” (*T. arboricola*), “C” (*T. corticola*), “I” (*T. impressa*), and “S” (*T. simplex*) (Helms et al., 2001; Helms, 2003). Each of these clades comprise several morphospecies.

According to the literature no sexual reproductive stages are known from representatives of the genera *Trebouxia* and *Asterochloris* and other genera of unicellular lichen photobionts within Trebouxiales (Friedl & Büdel, 1996). However, based on the observation of genetic diversity among conspecific *Trebouxia* photobionts in populations of *Letharia* species Kroken and Taylor (2000) concluded on recombination. This problem is discussed in Chapter 4.

1.3 Aims of the present investigation

Xanthoria parietina and closely related *Xanthoria* spp. (Teloschistaceae, Teloschistales) are the best investigated lichen-forming ascomycetes. Their thallus and ascomal ontogeny, primary and secondary metabolism, fine structure and carbohydrate transfer at the mycobiont-photobiont interface were studied in considerable detail (review: Honegger, 1990). The molecular and genetic basis of intra-thalline wall surface hydrophobicity (Scherrer et al., 2000, 2002; Scherrer & Honegger, 2003) and of mating systems were characterized (Honegger et al., 2004; Scherrer et al., 2005). The common and widespread *Xanthoria parietina* turned out to be a self-fertile species, which derived from cross-fertilized ancestors. Probably via cuttings of economic and ornamental woody perennials *X. parietina* was invading the Australian continent. The morphologically similar, very closely related, heterothallic *X. calcicola* and *X. ectaneoides* are restricted to Europe. All three species together are referred to as *X. parietina* complex. Very little is known about photobiont diversity within the Teloschistaceae and particularly within the genus *Xanthoria*. The present study aims at filling this gap.

Chaper 2 focusses on photobiont diversity in 12 *Xanthoria* species (including several South African endemic species), 7 *Xanthomendoza* species, *Teloschistes chrysophthalmus* and *Josefpoeltia boliviensis*. A high percentage of photobionts were isolated in sterile culture. Genetic diversity was analyzed using the following markers:

1. nuclear ribosomal ITS region (ITS1 & 2, 5.8S rDNA). ITS 1 & 2, as non-coding, rapidly evolving sequence areas, are among the most frequently used molecular markers in phylogenetic analyses (Rambold, 1998, Kroken & Taylor, 2000; Helms et al., 2001; Helms, 2003; Tiebell, 2001; Beck et al., 2002; Cordeiro et al., 2005; DePriest and Normore, 2004; Opanowicz and Martin, 2004; Yahr et al., 2004; Piercey-Normore, 2006).

2. the *rbc*L region, which is coding for part of the Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase (RuBisCO) holoenzyme. This centrally important enzyme in photosynthesis is expected to be highly conserved. The genome coding for *rbc*L sequences is located in the plastid.

Chapter 3 summarises newly found group I introns in the nuclear-encoded LSU and SSU rDNA regions of *Trebouxia* and *Asterochloris* species and their phylogenies.

Chapter 4 presents the first results ever obtained with fingerprinting techniques applied to sterile-cultured *Trebouxia* isolates from populations of *Xanthoria parietina*, using RAPD-PCR (randomly amplified polymorphic DNA) as a multilocus approach. The very few population studies published were concentrating on the fungal partner (Murtagh et al., 2002; Honegger et al., 2004; Lindblom & Ekman, 2005, 2006). So far no data were available on the genetic diversity at the subspecific level of photobionts within populations of lichen-forming fungi. Epiphytic and saxicolous samples of *Xanthoria parietina* were systematically collected in coastal, rural and urban sites in NW, SW and central France and on the University campus (Irchel-Park) in Zürich, Switzerland. As *Trebouxia* spp. do not sexually reproduce and, moreover, are assumed to be rare outside lichen thalli, we were interested to see whether populations of *Xanthoria parietina* contain one or few local genotpypes of *Trebouxia decolorans* or *T. arboricola*, or whether the algal inhabitants of these lichen thalli are genetically more diverse.

In Chapter 5 the photobionts of *Psoroglaena epiphylla* Lücking and *P. stigonemoides* (Orange) Henssen (Verrucariales) are identified and characterized. This small, but interesting side project was carried out because all molecular and microscopy tools and freshly collected material of these rare lichens were available and the problem is of general interest among lichenologists and phycologists. Verrucariales are known to associate with a wide range of photobionts (see 1.1.2). Based on light microscopy investigations the vivid green photobionts contained in the microfilamentous thalli of representatives of the genus *Psoroglaena* were hypothesized to belong to the prochlorophytes (Henssen, 1995). Prochlorophytes are the enigmatic group of marine cyanobacteria, which were speculated to be close to the ancestors of the green chloroplast of plants because they contain chlorophyll a and b, but no phycobilins (Lewin, 1976). As this would be the first terrestrial prochlorophytes ever found we were interested to investigate this symbiosis with ultrastructural techniques and with molecular markers (ITS and

*rbc*L regions). The photobionts of both *Psoroglaena* species turned out to be representatives of the green algal class Trebouxiophyceae.

Table 1.2 Main ultrastructural, biochemical and ecological characteristics of the five major green algal classes, as defined on the basis of molecular markers (modified after Graham & Wilcox, 2000)

class	flagellar/cytoskeletal apparatus	photorespiratory enzyme	mitosis	cytokinesis	primary habitat	life history
Prasinophyceae	cruciate roots, rhizoplasts, some with MLS, flagellar & body scales common	variable	variable	furrowing	marine	zygotic meiosis
Ulvophyceae	cruciate X-2-X-2 roots, CCW orientation, +/- body & flagellar scales, rhizoplast present	glycolate dehydrogenase	closed, persistent spindle	furrowing	marine	zygotic meiosis or alteration of generations or gametic meiosis
Trebouxiophyceae	cruciate X-2-X-2 roots, CCW orientation, no scales, rhizoplast present	glycolate dehydrogenase	semi-closed, non-persistent spindle	furrowing	freshwater or terrestrial	zygotic meiosis
Chlorophyceae	cruciate X-2-X-2 roots, CW or DO orientation, scales occur rarely, rhizoplast	glycolate dehydrogenase	closed, non-persistent spindle	furrowing, phycoplast, some with cell plate & plasmodesmata	freshwater	zygotic meiosis
Charophyceae	asymmetric roots, MLS body & flagellar scales usually present, rhizoplast rare	glycolate oxydase & catalase in peroxisome	open, persistent spindle	furrowing, some with cell plate, phragmoplast, & plasmodesmata	freshwater	zygotic meiosis

X: number of rootlets; CW: clockwise; CCW: counter clockwise; DO: directly opposed; MLS: multilayered structures;

Table 1.3 ITS sequences of *Trebouxia* and *Asterochloris* photobionts of lichen-forming fungi in publicly available databases

No. of ITS sequences	Lichen	Author
9	Physciaceae, other	Bhattacharya et al., 1996
6	Lecanorales	Beck et al., 1998
2	Lecanorales, Umbilicariales	Beck, 1999
16	Physciaceae	Friedl et al., 2000
15	*Letharia*	Kroken and Taylor, 2000
25	Physciaceae	Dahlkild et al., 2001
73	Cladoniaceae, *Anzina*, *Stereocaulon*	Piercey Normore and DePriest, 2001
24	Physciaceae	Helms et al., 2001
22	*Umbilicaria*	Romeike et al., 2002
3	*Rimularia*	de los Rios et al., 2002
3	*Fulgensia fulgida, Toninia sedifolia*	Beck et al., 2002
7	*Chaenotheca*	Beck, 2002
17	*Hypogymnia* and *Lecanora*	Helms et al., 2003
1	*Physcia*	Zhou et al., (unpubl.)
19	*Flavocetraria*	Opanowicz and Martin, 2004
24	Cladoniaceae	Yahr et al., 2004
46	*Cladonia*	Piercey-Normore, 2004
3	Free-living on rocks	Siddique and Arocena (unpubl.)
1	*Lecidea* (Lecideaceae)	De Los Rios et al., 2005
19	*Ramalina, Cladonia*	Cordeiro et al., 2005
45	*Lecanora*	Blaha et al., (unpubl.)
19	*Evernia mesomorpha*	Piercey-Normore, 2006
21	*Parmotrema tinctorum* (Parmeliaceae)	Ohmura et al., 2006
422		Total

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2 Green algal photobiont diversity (*Trebouxia* spp.) in representatives of Teloschistaceae (Lecanoromycetes, lichen-forming ascomycetes)

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2.1 Summary

The green algal photobionts of 12 *Xanthoria*, 7 *Xanthomendoza,* 2 *Teloschistes* species and *Josefpoeltia boliviensis* (all Teloschistaceae) were analysed. *Xanthoria parietina* was sampled on four continents. > 300 photobiont isolates were brought in sterile culture. The nuclear ribosomal internal transcribed spacer region (nrITS; 101 sequences) and the large subunit (*rbc*L) of the RuBiSco gene (54 sequences) of either whole lichen DNA or photobiont isolates were phylogenetically analysed. ITS and *rbc*L phylogenies were congruent although some subclades had low bootstrap supports. *Trebouxia arboricola, T. decolorans* and closely related, unnamed *Trebouxia* species, all belonging to clade A, were found as photobionts of *Xanthoria* species. *Xanthomendoza* species associated either with *T. decolorans* (clade A)*, T. impressa, T. gelatinosa* (clade I) or with an unnamed *Trebouxia* species*. Trebouxia gelatinosa* genotypes (clade I) were the photobionts of *Teloschistes chrysophthalmus, Telo. hosseusianus* and *Josefpoeltia boliviensis.* Only weak correlations between distribution patterns of algal genotypes and environmental conditions or geographical location were observed.

2.1.1 Key words:

Asterochloris, *Josefpoeltia boliviana*, nrITS, *rbc*L, *Xanthomendoza*, *Xanthoria, Teloschistes*, *Trebouxia*

2.2 Introduction

Lichens, as found in nature, are the symbiotic phenotype of lichen-forming fungi in association with their photoautotrophic partner. Species names of lichens refer to the fungal partner. Lichen photobionts, mostly green algae or cyanobacteria, very rarely Xanthophyceae or Phaeophyceae (Tschermak-Woess 1988; Peršoh *et al.* 2004), have their own names and phylogenies. Traditionally species of lichen-forming fungi were described on the basis of morphological and chemical characters (morpho- and chemospecies). Morphological criteria formed also the basis of species descriptions in lichen photobionts. In less than 3 % of the approximately 13'000 species of lichen-forming fungi has the photobiont ever been identified at species level (Honegger unpubl.); this estimate is based on Tschermak-Woess (1988) and on the recent literature.

As lichen-forming fungi do not easily re-lichenize under sterile culturing conditions the range of compatible photobiont taxa per lichen-forming fungal species can not be experimentally approached with re-lichenization experiments in the Petri dish. Instead, the photobiont of lichen specimens, as collected in the wild, is investigated. Traditionally, isolation and culturing under defined sterile conditions, followed by light or electron microscopic analysis and comparison with reference strains were used (Ahmadjian 1958; 1967; Tschermak-Woess 1988). Accordingly only few experts worldwide were able to identify the photobionts of lichen-forming fungi at species level. Since the advent of molecular techniques the time-consuming isolation and culturing was largely avoided; instead, photobiont-specific molecular markers applied to whole lichen DNA facilitated photobiont identification at species level (Kroken & Taylor 2000; Dahlkild *et al.* 2001; Helms *et al.* 2001; Piercey-Normore & Depriest 2001; Tibell 2001; Tibell & Beck 2001; Romeike *et al.* 2002; Helms 2003; Piercey-Normore 2004; 2006; Blaha *et al*. 2006). Based on increasing numbers of entries in databases novel insights in photobiont diversity and phylogenies were achieved. Isolation and culturing are, however, still crucial as reference material, for genetic analyses at subspecific level and for diverse experimental approaches.

The range of compatible partners per fungal species and their inter- and intraspecific diversity are ideally studied in a large set of samples from a wide geographic range. However, even the analysis of one or few samples gives valuable first insights in the taxonomic affiliation of compatible photobionts. The majority of morphologically advanced species of lichen-

forming fungi are moderately specific to specific as regards to their photobiont selection, i.e. associate with one or few species of green algae or cyanobacteria (Honegger 1993). A special situation occurs in cephalodiate taxa, an estimated 3-4% of all species of lichen-forming fungi, where a diazotrophic cyanobacterial partner is acquired in addition to the green algal primary photobiont (Tschermak-Woess 1988; Rambold *et al.* 1998; Peršoh *et al.* 2004). A lower specificity towards their photobiont was observed in few of the lichen-forming ascomycetes forming morphologically less advanced crustose thalli (Friedl 1987; Tschermak-Woess 1988; Helms 2003; Beck 2002; Blaha *et al*. 2006). An interesting situation was observed in the crustose genus *Chaenotheca* where multiple green algal switches may have occurred, resulting in a wide array of taxonomically very diverse photobionts associated with closely related fungal partners (Tibell 2001). The most common and widespread aerophilic unicellular green algae, often forming conspicuous green layers on bark or rock surfaces, are very rarely acceptable partners of lichen-forming fungi (Tschermak-Woess 1988; Peršoh *et al.* 2004).

More than 80% of lichen-forming fungi associate with green algal photobionts, representatives of the genera *Trebouxia* de Puym. and *Asterochloris* (Tscherm.-Woess) T. Friedl *ined*. (Trebouxiophyceae *sensu* Friedl 1995) being the most common and widespread partners in all climates (Ahmadjian 1988; Tschermak-Woess 1988; Rambold *et al.* 1998; Peršoh *et al.* 2004). Probably due to their ability to survive desiccation unharmed *Trebouxia* spp. are the photobionts of most lichen-forming fungi in climatically extreme habitats such as Antarctic, arctic, alpine or desert ecosystems, where the whole thallus is continuously subjected to drought and temperature extremes.

Sexually reproducing lichen-forming fungi are assumed to re-lichenize at each reproductive cycle, i.e. germinating asco- or basidiospores have to find a compatible photobiont. Contradictory views are found in the literature concerning the abundance of free-living *Trebouxia* cells and their availability for asco- or basidiospore-derived germlings of lichen-forming fungi. According to Ahmadjian (1988; 2001; 2002) *Trebouxia* species do not normally exist outside lichen thalli. Tschermak-Woess (1978) found free-living *Trebouxia* cells, but pointed out that they are rare in aerophilic algal communities. Bubrick *et al.* (1984) found free-living *Trebouxia* cells near thalli of *Xanthoria parietina*, and according to Mukhtar *et al.* (1994) *Trebouxia arboricola* de Puymaly is one of the most common colonizers of bare rock surfaces after fires in Israel. In a series of elegant *in* situ re-

lichenization studies, Sanders (2005) observed large numbers of free *Trebouxia* cells on plastic slides which had been exposed in oak trees (*Quercus ilex*) with lichen cover in Spain, and germ tubes of *Xanthoria parietina* ascospores contacting them. In the phycological literature the aerophilic *T. arboricola*, type species of the genus, is referred to as abundant and widespread on saxicolous and corticolous substrata in Europe (Ettl & Gärtner 1995; John *et al.* 2002; Rindi & Guiry 2003).

The complete internal transcribed spacer region (ITS 1, ITS 2 and 5.8S rDNA) was selected as main marker for investigating photobiont phylogenies. Additionally the *rbc*L locus was studied, which appears to be a highly suitable molecular marker for evaluating green algal phylogenies (McCourt *et al.* 1995; Nozaki *et al.* 1997; 2002; Sherwood *et al.* 2000). The *rbc*L gene encodes for the large subunit of Rubisco (ribulose-1,5-bisphosphate-carboxylase), which is the main protein present in the pyrenoid matrix of green algae, including *Trebouxia* spp. (Griffith 1970), where it was immunocytochemically located by Ascaso *et al.* (1995). 17 *Trebouxia* type strains were included in our study on *rbc*L phylogenies, which had been described as morphospecies by various investigators (Gärtner 1985b: Ettl & Gärtner 1984; Friedl 1989a).

The present study aims at exploring the identity, diversity and phylogeny of the photobiont in Teloschistaceae (Teloschistineae, Lecanoromycetes), the focus being on the genera *Xanthoria* and *Xanthomendoza*. Teloschistaceae are lichen-forming ascomycetes with world-wide distribution. They comprise species with a very wide geographic range such as the ubiquitous *X. elegans* and the very widespread *X. parietina* beside species with a small area of distribution such as the South African endemics *X. capensis, X. flammea* and *X. karrooensis*. Teloschistaceae are associated with trebouxioid green algal photobionts. Best investigated is the widely distributed type species of the genus, *Xanthoria parietina*, and the closely related European *X. calcicola* and *X. ectaneoides*, here referred to as the *X. parietina* complex (fungal phylograms in Scherrer & Honegger 2003; Eichenberger, unpubl.). According to the literature the *X. parietina* complex is associated with *Trebouxia arboricola* dePuymaly, *T. crenulata* Archibald*, T. decolorans* (Ahmad.) Arch. and *T. italiana* Arch. (Ahmadjian 1960; Gärtner 1985b; Honegger & Peter 1994; Beck *et al.* 1998).

The goals of the present study are 1) to evaluate photobiont diversity and phylogenies in a range of *Xanthoria* and *Xanthomendoza* spp.; 2) to explore the range of compatible photo-

bionts in a large sample of the *X. parietina* complex from worldwide locations. As *X. parietina* most likely was introduced to Australia and New Zealand (Galloway 1985; Rogers 1992) we were interested to see whether it associates with different photobionts in these areas than in Europe or North America. 3) to isolate *Trebouxia* photobionts of Teloschistaceae into sterile culture as reference strains and for diverse future investigations. Most of the corresponding fungal partners were brought in sterile culture (Honegger 2003), their taxonomic affiliation and phylogenies being analyzed in parallel experiments (PhD thesis of C. Eichenberger).

2.3 Materials and Methods

2.3.1 Lichen collection and storage

Freshly collected lichens were either immediately processed or stored, in desiccated state, at –20°C, where they stay viable for prolonged periods of time (Honegger 2003). Voucher specimens are kept at –20°C in our laboratory until completion of ongoing projects. Later they will be deposited in the herbarium of ETH Zürich (Z + ZT). Collectors and collecting sites are listed in Table 2.1. Few experiments were carried out with specimens from the lichen herbarium of the University of Graz, Austria. From a set of samples originating from the campus of the University of Zürich (Zürich-Irchel, numbers 319-320), the thalli were left *in situ* and only small fragments were removed after photographic documentation.

2.3.2 Photobiont isolation and culture

With a sterile platinum needle photobiont cells were scraped out of thalline margin of apothecia, or alternatively from the algal layer of lobes in samples with no or few ascomata. Photobiont cells were spread on the surface of agarized non-nutrient, mineral medium (Bold's basal medium [BBM] according to Deason & Bold 1960) contained in Petri dishes, with double amount of nitrogen and with 0.005% (w/v) Doxycycline (SIGMA) as an antibiotic. These plates were maintained at 15 ± 1°C at a 16:8h light/dark cycle at approximately 5 µE m^{-2} s^{-1} for 2-3 weeks until cells started to divide. All cultures were screened regularly; fungal contaminants were immediately cut out. Groups of dividing algal cells were either transferred to *Trebouxia* medium II according to Ahmadjian (1967), with only ¼ amount of glucose and casamino acids (Honegger 2004), and cul-

tured for 8-12 weeks, or left on BBM 2N. Most cultures are multi-cell isolates, cells originating from a very small area, but a few are single cell isolates. Approximately 300 sterile photobiont cultures from 12 identified and few unidentified *Xanthoria* species, 7 *Xanthomendoza* and 2 *Teloschistes spp.* were established. All isolates are stored in liquid nitrogen in our laboratory (Honegger *et al.* 1996). Reference strains obtained from the culture collection of algae at the Universities of Innsbruck, Austria (IB; Gärtner 1985a, b), and Göttingen are listed in Table 2.2.

Table 2.1 Photobiont isolates analyzed in present study

Lichen species	Collecting site	Country	Collector	Photobiont sp. †	Isolate†*	ITS acc #	rbcL acc #
Josefpoeltia parva (Räsänen) Fröden & L. Lindblom (syn. J. boliviana S.Y. Kondr. & Kärnefelt)	Tucuman	Argentina	B. Marrazzi, R. Vanni, G. Lopez	T. gelatinosa	L-447-I-P2	AM159212	
Teloschistes chrysophthalmus (L.) Th. Fr.	Canary Islands	E	R. Stalder	T. gelatinosa	P-270-I-a	AJ969579	AJ969640
Telo. hosseusianus Gyeln.	Tucuman	Argentina	B. Marrazzi, R. Vanni, G. Lopez	T. gelatinosa	L-447-t1	AM159211	
Xanthomendoza borealis (R. Sant. & Poelt) Søchting, Kärnefelt & S.Y. Kondr.		Greenland		Trebouxia sp.	G9306	AJ969505	AJ969662
Xm. borealis		Greenland		T. decolorans	G9307	AJ969506	
Xm. borealis		Greenland		T. decolorans	G9308	AJ969507	
Xm. fallax (Hepp) Arnold	California	USA	R. Robertson	T. impressa	L-46	AJ969525	AM158968
Xm. fallax	Chur	CH	U. Jauch	T. impressa	L-68	AJ969533	AM158969
Xm. fallax	Minnesota	USA	S. Scherrer	T. impressa	L-329-t1	AM159203	
Xm. fulva (Hoffm.) Poelt & Petut.	Sevan	Armenia	M. Kappali	T. decolorans	L-247-t1	AM159215	
Xm. hasseana (Räsänen) Søchting, Kärnefelt & S.Y. Kondr.	California	USA	R. Robertson	T. decolorans	P-69-I-a-Sc	AJ969534	AJ969652
Xm. hasseana	California	USA	S. Werth	T. decolorans	P-400-I-a-Sc	AM159210	
Xm. novozelandica (Hillmann) Søchting, Kärnefelt & S.Y. Kondr.	Roxburgh	NZ	D. J. Galloway	T. gelatinosa	L-66	AM159502	
Xanthomedoza sp.	Colorado	USA	C. Eichenberger	T. impressa	L-475-t2	AM159209	
Xanthomedoza sp.	Colorado	USA	C. Eichenberger	T. decolorans	L-477-t1	AM159207	
Xanthomedoza sp.	Colorado	USA	C. Eichenberger	Trebouxia sp.	L-478-t1	AM159208	
Xm. ulophyllodes (Räsänen) Søchting, Kärnefelt & S.Y. Kondr.	Minnesota	USA	S. Scherrer	T. impressa	P-330-I-b	AJ969605	AJ969636
Xm. weberii (S. Kondr. & Kärnefelt) L. Lindblom	Delaware	USA	O. Crichton	T. gelatinosa	P-57-I-a	AJ969532	AJ969642
Xm. weberii	Roussillon	F	R. Honegger	T. gelatinosa	L-114-t1	AM159214	
Xm. weberii	Massachusetts	USA	V. Ahmadjian	T. gelatinosa	P-350a-III	AM159213	
Xanthoria calcicola Oksner	Burgdorf	CH	R. Honegger	T. arboricola	P-44-I-a1	AJ969524	

X. calcicola	Lausanne	CH	S. Scherrer	T. arboricola	P-105-I-a	AJ969542	
X. calcicola	Avenches	CH	R. Honegger	T. arboricola	P-141-II	AJ969552	
X. calcicola	Hampshire	GB	P. J. James	T. arboricola	L-80	AJ969536	
X. candelaria (L.) Th. Fr.	Myvatn	IS	J. Achermann & G. Schuwey	T. decolorans	P-205-II-a	AJ969569	
X. candelaria	Nove Mesto	CZ	J. Lentjes	T. decolorans	L-264	AJ969576	AJ969655
X. capensis Kärnefelt, Arup & L. Lindblom	Cape Town	ZA	A. Möhl	T. arboricola	P-306-I-a	AJ969591	
X. ectaneoides (Nyl.) Zahlbr.	Cornwall	GB	J. M. Gray	T. arboricola	P-83-I-a	AJ969611	
X. ectaneoides	Mt. Eros, Hydra	GR	O.W. Purvis	T. arboricola	P-85-II-a	AJ969537	
X. ectaneoides	Hydra	GR	O.W. Purvis	T. arboricola	P-86-I-b	AJ969538	AJ969666
X. ectaneoides	Bretagne	F	R. Honegger	T. arboricola	P-158-IV-mc	AJ969560	
X. ectaneoides	Sicily	I	R. Honegger	T. arboricola	L-43	AJ969523	
X. ectaneoides	Karthago	TN	U. Zippler	T. arboricola	P-174-II-adA	AJ969565	
X. elegans (Link) Th. Fr.	Manasulu	Nepal	F. Rutschmann	T. decolorans	L-269	AJ969578	
X. elegans	Gemmi Pass	CH	H. P. Schob	Trebouxia sp.	L-398-t1	AM159204	
X. elegans	Bishkek	KS	L. E. Tapernoux	Trebouxia sp.	L-459-t1	AM159206	
X. flammea (L. f.) Hillmann	West coast	ZA	H.P. Ruffner & E. Ruiz	T. arboricola	L-101	AJ969540	AJ969664
X. karrooensis S.Y. Kondr. & Kärnefelt	Western Cape	ZA	H. Gansner	T. arboricola	P-360-I	AM159216	
X. ligulata (Körb.) P. James	South Island	NZ	W. Malcom	T. arboricola	P-17-II-a	AJ969519	
X. ligulata	South Island	NZ	J. Bannister & A. Knight	T. arboricola	P-53-I-a	AJ969528	AJ969670
X. ligulata	South Island	NZ	J. Bannister & A. Knight	T. arboricola	P-54-II-a	AJ969530	
X. parietina (L.) Th. Fr.	Tasmania	AUS	G. Kantvilas	T. decolorans	P-10-I-a	AJ969515	AJ969659
X. parietina	Tasmania	AUS	G. Kantvilas	T. arboricola	L-11-II-a	AJ969516	
X. parietina	Port Fairy	AUS	U. & R. Stidwill	T. decolorans	P-133-I-a	AJ969551	AM158961
X. parietina	Barossa Valley	AUS	J. Pokorny	T. decolorans	L-275-II	AJ969580	
X. parietina	Canberra	AUS	J. Pokorny	T. arboricola	P-276-I-a	AJ969581	
X. parietina	Grampians	AUS	J. Pokorny	T. arboricola	L-277-I	AJ969582	
X. parietina	Roxburgh	NZ	D.J. Galloway	T. decolorans	L-1-II-A	AJ969508	
X. parietina	South Island	NZ	J. Bannister & A. Knight	T. decolorans	L-51-I	AJ969527	
X. parietina	Oregon	USA	B. Mc Cune	T. decolorans	P-6-I-a	AJ969511	
X. parietina	California	USA	R. Robertson	T. decolorans	L-8	AJ969513	
X. parietina	California	USA	R. Robertson	T. decolorans	L-9	AJ969514	

X. parietina	Maine	USA	J. Hinds	T. arboricola	L-26	AJ969521	
X. parietina	Maine	USA	J. Hinds	T. decolorans	P-28-I-a	AJ969522	
X. parietina	Massachusetts	USA	V. Ahmadjian	T. arboricola	L-348	AJ969607	
X. parietina	Oslo	N	T. Tønsberg	T. decolorans	L-16-I-A	AJ969517	
X. parietina	Northamptonshire	GB	J. J. Pittet	T. arboricola	P-18-I-a	AJ969520	AJ969647
X. parietina	Gotland	S	S. Scherrer	T. decolorans	P-97-I-a	AJ969539	AM158965
X. parietina	Canary Islands	E	M. Trembley	T. decolorans	P-104-II-a	AJ969541	AJ969651
X. parietina	Madrid	E	R. Schönthal	T. decolorans	L-265-II	AJ969577	
X. parietina	Mallorca	E	M. Trembley	T. decolorans	P-280-II-a-Sc	AJ969583	AJ969660
X. parietina	Mallorca	E	M. Trembley	T. decolorans	P-281-I-a-Sc	AJ969584	
X. parietina	Mallorca	E	M. Trembley	T. decolorans	P-282-I-a-Sc	AJ969585	
X. parietina	Paphos	CY	A. Birchmeier	T. arboricola	P-5-I-a-A	AJ969510	
X. parietina	Keflavik	IS	J. Achermann & G. Schuwey	T. arboricola	P-198-II-a	AJ969568	
X. parietina	Thingvellir	IS	J. Achermann & G. Schuwey	T. arboricola	P-210-I-a	AJ969570	AJ969648
X. parietina	Sevan	Armenia	M. Käppeli	T. decolorans	P-246-I-a-Sc	AJ969574	
X. parietina	Sevan	Armenia	M. Käppeli	T. decolorans	P-249-I-a	AJ969575	
X. parietina	Bretagne	F	R. Honegger	T. arboricola	P-7-I-a	AJ969512	AJ969646
X. parietina	Cerdagne	F	R. Honegger	T. decolorans	P-116-II-b-A	AJ969543	
X. parietina	Roussillon	F	R. Honegger	T. decolorans	P-120-I-bd	AJ969544	
X. parietina	Roussillon	F	R. Honegger	T. decolorans	P-121-a1Dark	AJ969547	
X. parietina	Roussillon	F	R. Honegger	T. decolorans	P-121-a1Light	AJ969548	
X. parietina	Roussillon	F	R. Honegger	T. decolorans	P-121-II-cd	AJ969550	AM158967
X. parietina	Roussillon	F	R. Honegger	T. decolorans	P-121-II-gd		AJ969656
X. parietina	Bourgogne	F	R. Honegger	T. decolorans	P-144-III-bd	AJ969554	
X. parietina	Bourgogne	F	R. Honegger	T. decolorans	P-145-I-dj	AJ969559	
X. parietina	Bretagne	F	R. Honegger	T. decolorans	P-164-I-a	AJ969561	
X. parietina	Bretagne	F	R. Honegger	T. decolorans	P-164-IX-a-2	AJ969563	
X. parietina	Corsica	F	L. Walthert & K. Boschi	T. arboricola	P-218-I-a	AJ969573	
X. parietina	Zürich	CH	S. Nyati & R. Honegger	T. decolorans	P-319-I-g	AJ970889	AM159504
X. parietina	Zürich	CH	S. Nyati & R. Honegger	T. decolorans	P-319-II-c1	AJ969596	
X. parietina	Zürich	CH	S. Nyati & R. Honegger	T. decolorans	P-319-IV-c2	AJ969598	
X. parietina	Zürich	CH	S. Nyati & R. Honegger	T. decolorans	P-320-II-c	AJ969601	
X. parietina	Zürich	CH	S. Nyati & R. Honegger	T. decolorans	P-320-II-f	AJ969603	AM158963

X. parietina	Zürich	CH	S. Nyati & R. Honegger	T. arboricola	P-320-III-a	AJ969604	AJ969668
X. parietina	Nekrasova	RUS	T. Horath	T. decolorans	P-191-I-a	AJ969567	AJ969654
X. parietina	Stavropol	RUS	K. Bouke	T. decolorans	P-213-I-a	AJ969571	
X. parietina	Cape Point	ZA	H. Gansner	T. decolorans	L-356	AJ969608	
X. polycarpa (Hoffm.) Th. Fr. ex Rieber	Otago	NZ	J. Bannister & A. Knight	T. arboricola	P-48-III-a	AJ969526	
X. polycarpa	Oregon	USA	B. Mc Cune	T. decolorans	P-56-II-a	AJ969531	
X. polycarpa	California	USA	R. Robertson	T. decolorans	P-71-II-b	AJ969535	
X. polycarpa	Zürich	CH	R. Honegger	T. decolorans	P-215-I-a	AJ969572	AM158962
Xanthoria sp.	Adelaide	AUS	M. Federer	T. decolorans	L-184	AJ969566	
Xanthoria sp.	Sevan	Armenia	M. Käppeli	T. decolorans	L-243-t1	AM159202	
Xanthoria sp.	Tilos	GR	U. & R. Stidwill	T. decolorans	P-287-VI-b	AJ969586	AJ969649
Xanthoria sp.	Tilos	GR	U. & R. Stidwill	T. decolorans	P-288-I-a	AJ969587	AJ969650
Xanthoria sp.	Tilos	GR	U. & R. Stidwill	T. decolorans	P-303-III-a	AJ969589	AJ969667
Xanthoria sp.	Tilos	GR	U. & R. Stidwill	T. decolorans	P-304-I-a	AJ969590	
Xanthoria sp.	Canary Islands	E	C. Eichenberger	T. arboricola	L-337	AJ969606	AJ969645
X. sorediata (Vain.) Poelt	Langweis	CH	S. Scherrer & C. Eichenberger	Trebouxia sp.	L-454-t1	AM159205	
X. turbinata Vain	Port Nolloth	ZA	R. Dudler	T. arboricola	P-3-I	AJ969509	AJ969669

† Photobiont species determined based on ITS and rbcL sequence data where available, †* P photobiont isolated, L whole lichen DNA was used for PCR amplification and sequencing where axenic cultures could not be established followed by voucher number, thallus number and apothecia or lobe number. Sc single cell isolate. G lichen material obtained from the herbarium of the University of Graz.

Table 2.2 List of reference *Trebouxia* strains and their ITS and *rbc*L accession numbers

Photobiont species*	Strain †*	Lichen species	Collecting site	rbcL acc #	ITS acc #	Reference **
T. aggregata (Archibald) Gärtner	UTEX 180/IB 325	*Xanthoria sp.* (Fr.) Th. Fr.	Delft, Netherlands	AJ969643	unpublished[1]	This study
T. anticipata Ahmadjian ex Archibald	UTEX 903/IB 340	*Parmelia rudecta* Ach.	USA	AJ969638		This study
*T. arboricola** Puymaly	SAG 219-Ia ‡	Free living?	MA, USA	AM158960	Z68705	Bhattacharya et al. (1996)
T. arboricola	M-92.025C1	*Xanthoria parietina* (L.) Th. Fr.	Munich, Germany		AJ007387	Beck et al. (1998)
T. asymmetrica Friedl & Gärtner	B207	*Toninia sedifolia* (Scop.) Timdal	France		AF344177	Beck et al. (2002)
*T. corticola** (Archibald) Gärtner	UTEX 909	Free living?	MA, USA		AJ249566	Friedl et al. (2000)
*T. crenulata** Archibald	CCAP 219-2/IB 359	*X. aureola* (*sub X. calcicola* Oksner)	England	AJ969639	unpublished[1]	This study
*T. decolorans** Ahmadjian	UTEX 901/IB 327	*X. parietina* (L.) Th. Fr.	USA	AJ969657	unpublished[1]	This study
*T. excentrica** Archibald	UTEX 1714/IB 345	*Stereocaulon dactylophyllum* Flörke	USA	AJ969629		This study
*T. flava** Archibald	UTEX 181/IB 346	*Physcia pulverulenta* (Schreb.) Hampe	Delft, Netherlands	AJ969637	AF242467	Kroken & Taylor (2000)
*T. galapagensis** (Hildreth & Ahmadjian) Gärtner	UTEX 2230	*Ramalina sp.* Ach.	Galapagos Islands		AJ249567	Friedl et al. (2000)
*T. gelatinosa** Ahmadjian ex Archibald	UTEX 905/IB 347	*Parmelia caperata* (L.) Ach.	USA	AJ969641		This study
T. gelatinosa	87.072B1	*Parmelia subrudecta* Nyl.			AJ249575	Friedl et al. (2000)
*T. gigantea** (Hildreth & Ahmadjian) Gärtner	UTEX 2231	*Caloplaca cerina* (Ehrh. ex Hedw.) Th. Fr.	Ohio, USA		AF242468	Kroken & Taylor (2000)
*T. higginsiae** (Hildreth & Ahmadjian) Gärtner	UTEX 2232/IB 335	*Buellia straminea* Tuck.	Galapagos Islands		AJ249574	This study; Friedl et al. (2000)
T. impressa Ahmadjian	8.70E+02	*Parmelina carporrhizans* (Taylor) Poelt & Vizda			AJ249570	Friedl et al. (2000)
*T. incrustata** Ahmadjian ex Gärtner	UTEX 784	*Lecanora dispersa* (Pers.) Röhl.	USA		AJ293795	Helms et al. (2001)
*T. jamesii** (Hildreth & Ahmadjian) Gärtner	UTEX 2233/IB 336	*Schaereria tenebrosa* (Flot.) Hertel & Poelt	England	AJ969663	unpublished[1]	This study
*T. potteri** Ahmadjian ex Gärtner	UTEX 900/IB 332	*Lecanora rubina* (Vill.) Ach.	MA, USA	AJ969635	AF242469	Kroken & Taylor (2000)
*T. showmanii** (Hildreth & Ahmadjian) Gärtner	UTEX 2234/IB 337	*Lecanora hageni* (Ach.) Ach.	USA	AJ969661	AF242470	Kroken & Taylor (2000)
T. simplex Tschermak-Woess	TW-1A2	*Chaenotheca chrysocephala* (Turner ex Ach.) Th. Fr.	Austria		unpublished[1]	
Asterochloris (Tschermak-Woess) T. Friedl (ined.) (isolates are kept under the genus name *Trebouxia* de Puymaly in culture collections)						
*A. erici** (Ahmadjian) T. Friedl (ined.)	UTEX 910/IB 342	*Cladonia cristatella* Tuck.	USA	AJ969631		This study
A. erici	UTEX 912	*Cladonia cristatella* Tuck.	MA, USA		AF345441	Piercey-Normore et al. (2001)
A. glomerata (Ahmadjian) T. Friedl (ined.)	UTEX 894/IB 349	*Stereocaulon evolutoides* (H. Magn.) Frey	MA, USA	AJ969633		This study

A. glomerata	UTEX 897	*Stereocaulon pileatum* Ach.	Princeton, USA		AF345405	Piercey-Normore et al. (2001)
*A. italiana** (Archibald) T. Friedl (ined.)	CCAP 219-5b/IB 358	*X. parietina*(L.) Th. Fr.	Italy	AJ969632		This study
*A. magna** (Archibald) T. Friedl (ined.)	UTEX 67	*Cladonia sp.* P. Browne	Delft, Netherlands		AF345423	Piercey-Normore et al. (2001)
A. magna	UTEX 902/IB 354	*Pilophorus acicularis* (Ach.) Th. Fr.	USA	AJ969630		This study
*A. pyriformis** (Archibald) T. Friedl (ined.)	UTEX 1713/IB 356; UTEX 1712/IB 355	*Stereocaulon pileatum* Ach.; *Cladonia squamosa* (Scop.) Hoffm.	USA	AJ969634	AF345407	Piercey-Normore et al. (2001)

* type strains are indicated with an asterisk; †*UTEX- Algal Culture Collection at University of Texas; IB- Algal Culture Collection at University of Innsbruck, SAG- Algal Culture Collection at University of Göttingen; other strains are in the private culture collections; ‡ Type species of the genus *Trebouxia de Puymaly*; ** references applicable only for already published nrITS sequences, all *rbc*L sequences were generated in present study; [1] ITS sequences labelled “unpublished” were generated by Thomas Friedl and were kindly provided by himself and Gert Helms for comparison.

Table 2.3 List of primers used in present study

Primer	Target	Sequence (5'--> 3')	Reference
ITS4 (rev)	LSU	TCCTCCGCTTATTGATATGC	White et al. (1990)
ITS5 (fwd)	SSU	GGAAGTAAAAGTCGTAACAAGG	White et al. (1990)
AL1500bf (fwd)	SSU	GATGCATTCAACGAGCCTA	Helms et al. (2001)
LR3 (rev)	LSU	CCGTGTTTCAAGACGGG	Friedl et al. (1997)
TreSeq1 (fwd)	5.8 s nrDNA	CAACTCTCAACAACGGATATC	This study
TreSeq2 (rev)	5.8 s nrDNA	GACGCTGAGGCAGACATGCTC	This study
TreSeq3 (rev)	5.8 s nrDNA	CCGAAGCCTCGAGCGCAATTT	This study
rbcLfwd (fwd)	*rbc*L	GAMACTGATATTCTTCTTGCAGC	This study
rbcLrev (rev)	*rbcL*	GCAGCTAATTCAGGACTCCA	This study
rbcL1 (fwd)	*rbc*L	CGTGGTGGTTTAGATTTTAC	This study
rbcL2 (rev)	*rbc*L	ATTTGCGTTGACGACCATGA	This study
rbcL3 (rev)	*rbc*L	ATTTACGTTGTCGTCCATGT	This study
rbcL4 (fwd)	*rbc*L	GCAGCDTTYCGTATGACTCCTCAA	This study

2.3.3 DNA extraction, PCR amplification and Sequencing

Genomic DNA was isolated and purified using GFX PCR, DNA and Gel Band Purification Kit (Amersham Biosciences). Algal isolates or lichen samples, respectively, were frozen in liquid nitrogen prior to grinding. After addition of 100 µl of capture buffer to ground material, the samples were incubated at 60°C for 10 minutes and subsequently centrifuged. The supernatant was transferred to GFX column, which had been preloaded with 100 µl of capture buffer, incubated for 3 minutes at RT, centrifuged and washed with 500 µl of washing buffer. The DNA was eluted in 50 µl of elution buffer (10 mM Tris-HCL, pH 8.0) and stored at 4°C.

2.3.4 ITS amplification

The nuclear ribosomal ITS region (ITS1, 5.8S rDNA and ITS2) of algal isolates was amplified using primer pair ITS5 and ITS4 as described by White *et al.* (1990). For whole lichen DNA, forward primer AL1500bf (Helms *et al.* 2001) and reverse primer LR3 (Friedl & Rokitta 1997) were used with following PCR conditions: initial denaturation at 95°C for 3 min, followed by 32 cycles (94°C for 40 s, 50°C for 40 s, and 72°C for 80 s), with final extension at 72°C for 10 min. Internal primers at 5.8S rDNA were newly designed (Table 2.3). PCR products were purified with GFX PCR, DNA and Gel Band Purification Kit (Amersham Biosciences, NJ, USA) following the standard protocol provided by the manufacturers and sequenced directly.

When direct sequencing did not give satisfactory results, the samples were cloned using pGEM®-T Easy Vector System (Promega Corp., WI, USA) and competent XL10-Gold® *Escherichia coli* cells (Stratagene, CA, USA). Plasmid DNA was isolated using GFX™ Micro Plasmid Prep Kit (Amersham Biosciences, NJ, USA).

2.3.5 *rbc*L amplification

Six different primers were newly designed for amplification and sequencing of the large subunit (*rbc*L) of plastid gene ribulose-1, 5-biphosphate carboxylase/oxygenase. PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 30 cycles (95°C for 45 s, 52°C for 60 s, and 72°C for 80 s), with final extension at 72°C for 10 min.

2.3.6 Sequencing

Purified PCR fragment (10-20 ng DNA) or plasmid (150-300 ng DNA) was used for sequencing in 10 µl reaction mix containing 120 nM primer, 0.8 µl BigDye Terminator Mix V3.1, and 1X reaction Buffer. Amplification conditions were as follows: initial denaturation at 94°C for 2 min, followed by 60 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 3 min (0.9°C/s ramp). The products were analyzed on an Applied BioSystem/HITACHI ABI 3730 DNA Analyzer.

2.3.7 Phylogenetic analysis

Sequences were analyzed with Sequencher™ 4.2.2 (Gene Codes Corp. Ann Arbor, USA) and aligned automatically with Clustal X 1.81 (Thompson *et al.* 1997), with gap opening penalty 10.0 and gap extension penalty 0.20. Aligned sequences were imported in MacClade 4.06 (Maddison & Maddison 2002) and manually aligned. Phylogenetic analysis was carried out using Paup 4.0b10 (Swofford 1998) by Maximum likelihood (ML), Maximum Parsimony (MP) and Neighbor joining (NJ) methods. Jackknife values for 500 replicates were calculated separately by MP and NJ analyses. ITS analyses were carried out with complete ITS1, ITS2 and 5.8S rDNA sequences. Intron sequences were cut out from the nrITS alignment since these were present in only 25% of newly generated ITS sequences. In ITS analyses *T. simplex* sequences were used as outgroup while in *rbc*L analyses *Asterochloris* sequences were used as outgroup.

2.4 Results

2.4.1 Isolation and culturing

In the course of ongoing projects about 300 photobiont isolates were obtained from most of the freshly collected lichen specimens, with or without prior storage at -20°C. On non-nutrient mineral medium (BBM) all isolates grew well, albeit more or less slowly, and kept their green coloration. There was neither any evidence of bleaching under the light conditions provided, nor of any dependence on external nutrient supply, as suggested by Ahmadjian (1960; 2001; 2002). In our laboratory the type strain of *T. decolorans* retained its color with the same intensity after 4 months culturing on either BBM 2N or *Trebouxia* ¼ media. Different growth rates were observed among different isolates, partly even among isolates from samples collected next to each other (e.g. among isolates 319 and

320). Only one algal isolate was normally taken per lichen sample. All except one isolate were phenotypically homogenous, and RAPD-PCR analyses of diverse subsamples per isolate turned out to be homogenous (5 subsamples each of 5 isolates tested with 3 primers, data not shown). However, two phenotypically different isolates (P-121-I-a, either light green, or dark green to brownish) were obtained from the same apothecium of a *X. parietina* sample. As concluded from ITS phylogenetic analyses both isolates represented different genotypes of the same algal species (*T. decolorans*) (see Fig. 2.1).

2.4.2 ITS phylogeny

A total of 124 photobiont nrITS sequences were obtained in this study, originating from 12 *Xanthoria* species, 7 *Xanthomendoza* species, 2 *Teloschistes* species, *Joselfpoeltia boliviensis* and few unnamed *Xanthoria* and *Xanthomendoza* samples. 23 nearly identical sequences obtained from common localities have been omitted from phylogenetic analyses therefore only 101 newly generated sequences were used for analyses. A total of 781 characters were included in nrITS (ITS1, 5.8S rDNA and ITS2) phylogenetic analysis, 358 of which were constant, 118 were variable but uninformative, and 305 were parsimony informative. Tree topologies for main clades were identical in ML, MP and NJ analyses. Only one most likely tree resulted in ML parsimony analyses (Fig. 2.1). In 32 out of 124 ITS sequences a longer ITS fragment was found due to a group I intron at position 1512 as described by Bhattacharya *et al.* (1996; 2002) and Helms *et al.* (2001). Intron sequences were removed from ITS alignments prior to phylogenetic analysis.

The major clades in ITS phylogeny were in accordance with the *Trebouxia* clade system as proposed by Helms *et al.* (2001) and Helms (2003). Clade A includes *T. arboricola* (including *T. aggregata* and *T. crenulata*), *T. decolorans, T. asymmetrica*, *T. showmanii, T. incrustata* and *T. jamesii*. Clade A was subdivided into an *arboricola* cluster (subclades Aa and Ab) and a *decolorans* cluster (subclades Ac, and Ad); unnamed *Trebouxia* species form subclade Ae. Clade I comprised the *impressa* (subclade Ia) and *gelatinosa* (subclade Ib) clusters.

ML phylogram
ITS1, 5.8S rDNA and ITS2

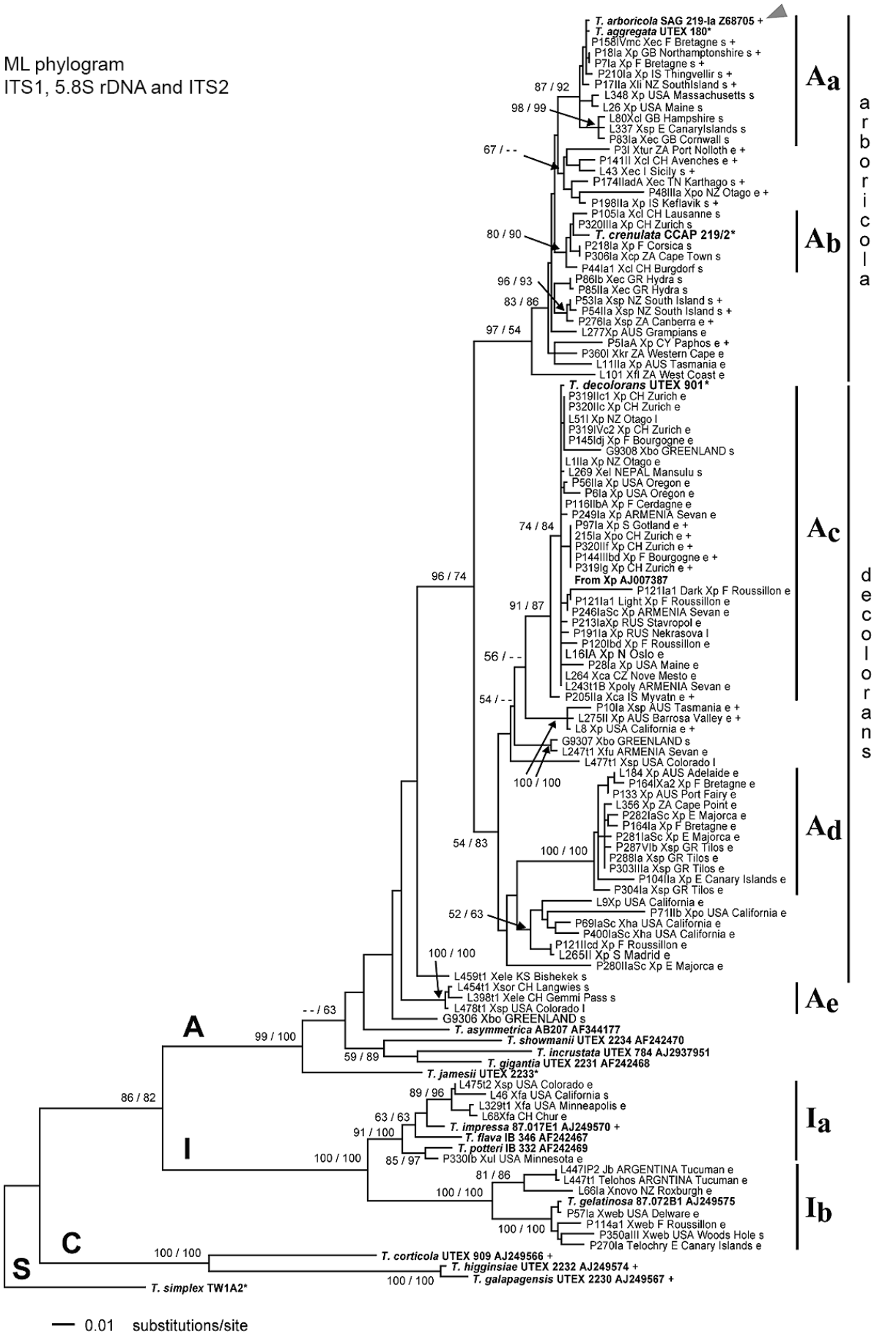

Figure 2.1 ML phylogram of nrITS region (combined ITS1, ITS2 and 5.8S rDNA). Jackknife values calculated separately for 500 replicates by MP (first number) and NJ (second number) analyses and indicated at the nodes. *Trebouxia simplex* sequences were used as outgroup. Arrowhead points to the type species of the genus *Trebouxia de Puymaly*. Abbreviations used: *Xanthoria*: *Xbo: Xanthoria borealis, Xca: X. candelaria, Xcl: X. calcicola, Xcp: X. capensis*, *Xec: X. ectaneoides, Xel: X. elegans, Xfl: X. flammea, Xli: X. ligulata, Xp: X. parietina, Xpo: X. polycarpa, Xsp:* unidentified *Xanthoria* or *Xanthomendoza sp, Xtu: X. turbinata. Xanthomendoza: Xfa: Xanthomendoza fallax, Xfu: Xm. fulva, Xha: Xm. hasseana, Xnovo: Xm. novozelandica, Xul: Xm. ulophyllodes, Xweb: Xm weberi; Teloschistes: Telochry: Teloschistes chrysophthalmus*, *Telohos: Telo. hosseusianus; Josefpoeltia: Jb: Josefpoeltia boliviensis.* Letters A, C, I & S indicate *Trebouxia* clades as proposed by Helms 2003). P: photobiont isolated, L: whole lichen DNA used for amplification, e: epiphytic; s: saxicolous; l: lignicolous/ corticolous; +: sequence contained 1512 intron. Sequences obtained from databases are in bold and indicated with strain number and accession number; *: unpublished sequence provided by G. Helms. Arrowhead indicates type species of the genus.

Photobionts of all identified and unidentified *Xanthoria spp*. analyzed in this study belonged either to *T. decolorans*, *T. arboricola* or closely related, unnamed *Trebouxia sp*. within clade A (Fig 2.1). Best represented in our sample set was *X. parietina,* with 49 ITS sequences from specimens collected on 4 continents. Photobionts of *Xanthomendoza* species belonged to either the *arboricola* (A) or *impressa* (I) clades.

Photobionts of eight identified and four unnamed *Xanthoria* species were represented in the *arboricola* cluster (subclades Aa and Ab), which is characterized by a 28 nucleotides long insert within ITS1 (Helms *et al.* 2001). Subclade Aa has jackknife support of 87% (MP) and 92% (NJ). It includes the type species of the genus, *Trebouxia arboricola* (strain SAG 219-I-a, arrowhead), *T. aggregata* (Archi.) Gärtner (UTEX 180) and photobiont isolates of *X. calcicola, X. ectaneoides, X. ligulata, X. parietina,* and an unidentified *Xanthoria* species, phenotypically resembling *X. parietina* (L-337, Canary Islands). Subclade Ab of the *arboricola* cluster includes *T. crenulata* (strain CCAP 219/2), photobionts of *X. calcicola, X. parietina,* and of the South African endemic species *X. capensis*. Photobiont isolates of *X. turbinata*, *X. calcicola*, *X. ectaneoides*, *X. polycarpa* and *X. parietina* formed a cluster which was only weakly supported in MP analysis and not supported by NJ analysis. Photobionts of unnamed *Xanthoria* species from New Zealand and Australia made a small cluster with very high support but they went together with photobionts of *X. flammea* (ZA), *X. ectaneoides*, *X. karrooensis* and *X. parietina* forming a basal unresolved part without strong support. Subclade Ac, being part of the *decolorans* cluster, has high jackknife supports in MP and NJ analyses (91% and 87% respectively). It includes *T. decolorans* (UTEX 901) and photobiont sequences of *X. parietina* (CH, NZ, F, RUS, S, USA), *X. candelaria* (CZ, IS), *X. elegans* (Nepal), *X. polycarpa* (Armenia, CH, USA) and *X. borealis* (Greenland). The ITS sequence of *X. parietina* photobiont identified as *T. arboricola* (AJ007387) by Beck *et al.*

(1998) also goes in this clade. Subclade Ad, also being part of the *decolorans* cluster, is very well supported (100%). It comprised photobiont sequences of four unidentified *Xanthoria species* clustering within the *X. parietina* complex (GR) along with photobiont sequences of *X. parietina* s. str. (AUS, F, E, USA & ZA). The phylogenetic position of several photobionts, which cluster outside subclades Ac and Ad, could not be properly resolved. This part of the tree includes the photobionts of *Xanthomendoza fulva* (Armenia), *Xm. hasseana* (USA), *X. borealis* (Greenland), *X. candelaria* (IS), *X. parietina* (AUS, F, E and USA), *X. polycarpa* (USA), and unidentified *X. sp.* (AUS, USA). Subclade Ae has high jackknife support in both ML and NJ analyses (100%) and includes photobiont sequences of *X. elegans* (CH), X*. sorediata* (CH), and an unidentified *Xanthomendoza* sp. (USA). This ITS subclade Ae most likely represents a cryptic *Trebouxia sp.* Exact phylogenetic position of photobionts of *X. borealis* (G 9306; Greenland) and *X. elegans* (KS) could not be resolved.

Subclade Ia has a very high bootstrap support (100%). This might be partly due to the small sample size. It includes *T. impressa*, *T. potteri*, *T. flava* and photobiont sequences of *Xanthomendoza fallax* (CH, USA), *Xm. ulophyllodes* (USA) and an unidentified *Xanthomendoza sp.* (USA). Helms (2003) found the authentic strain of *T. impressa* (UTEX 893) to be very similar to *T. potteri* (UTEX 900) and most probably conspecific with *T. flava* (UTEX 181), as inferred from ITS p-distances. Therefore, all our isolates in this cluster are referred to as *T. impressa*. Subclade Ib, the *gelatinosa* cluster, comprises two well separated groups, one of them harbouring the type strain of *T. gelatinosa* beside photobiont sequences of *Teloschistes chrysophthalmus* (E), and *Xanthomendoza weberii* (F, USA), the other photobiont isolates of *Xanthomendoza novozelandica* (NZ), *Teloschistes hosseusianus* (Argentina) and *Josefpoeltia boliviensis* (Argentina). *Telo. hosseusianus* and *J. boliviensis* grew side by side and were locally overgrowing each other; it is interesting to see that they associate with the same photobiont.

2.4.3 *rbc*L phylogeny

A total of 1155 characters were included in phylogenetic analyses of the *rbc*L gene, 925 of which were constant, 34 variable but uninformative, and 196 were parsimony informative. ML (Fig. 2.2), MP and NJ analyses resulted in similar tree topologies. *Asterochloris* sequences formed an outgroup. *rbc*L phylogeny was largely congruent with ITS phylog-

eny. *T. arboricola* (SAG 219-Ia) and *T. aggregata* (IB 325) were part of subclade Aa (bootstrap support 99%) while *T. crenulata* (IB 359) was part of subclade Ab, as was also the case in the ITS phylogram. The *rbc*L clade Ab had very low (57 in NJ) or no jackknife support (MP). Photobiont isolates of *X. flammea* (ZA), *X. ectaneoides* (GR) and of an unidentified *Xanthoria sp.* (NZ) fell outside subclade Ab. Deduced amino acid sequences within subclade Aa were identical and differed from subclade Ab sequences only marginally (data not shown). Subclade Ac with low jackknife support (53, 64) comprised *T. decolorans* (IB 327) along with isolates, which were also part of subclade Ac in ITS phylogeny. Subclade Ad, which is highly supported in the ITS phylogram is not supported in *rbc*L phylogeny (dotted line). The photobiont isolates of *Xm. fallax* (CH, USA) and *Xm. ulophyllodes* (USA) clustered with *T. impressa, T. potteri* and *T. flava* in subclade Ia, which was very well supported (100%). All *rbc*L sequences in subclade Ib, including type strains *T. gelatinosa* and *T. anticipata*, were nearly identical. Six representatives of the genus *Asterochloris* formed the outgroup.

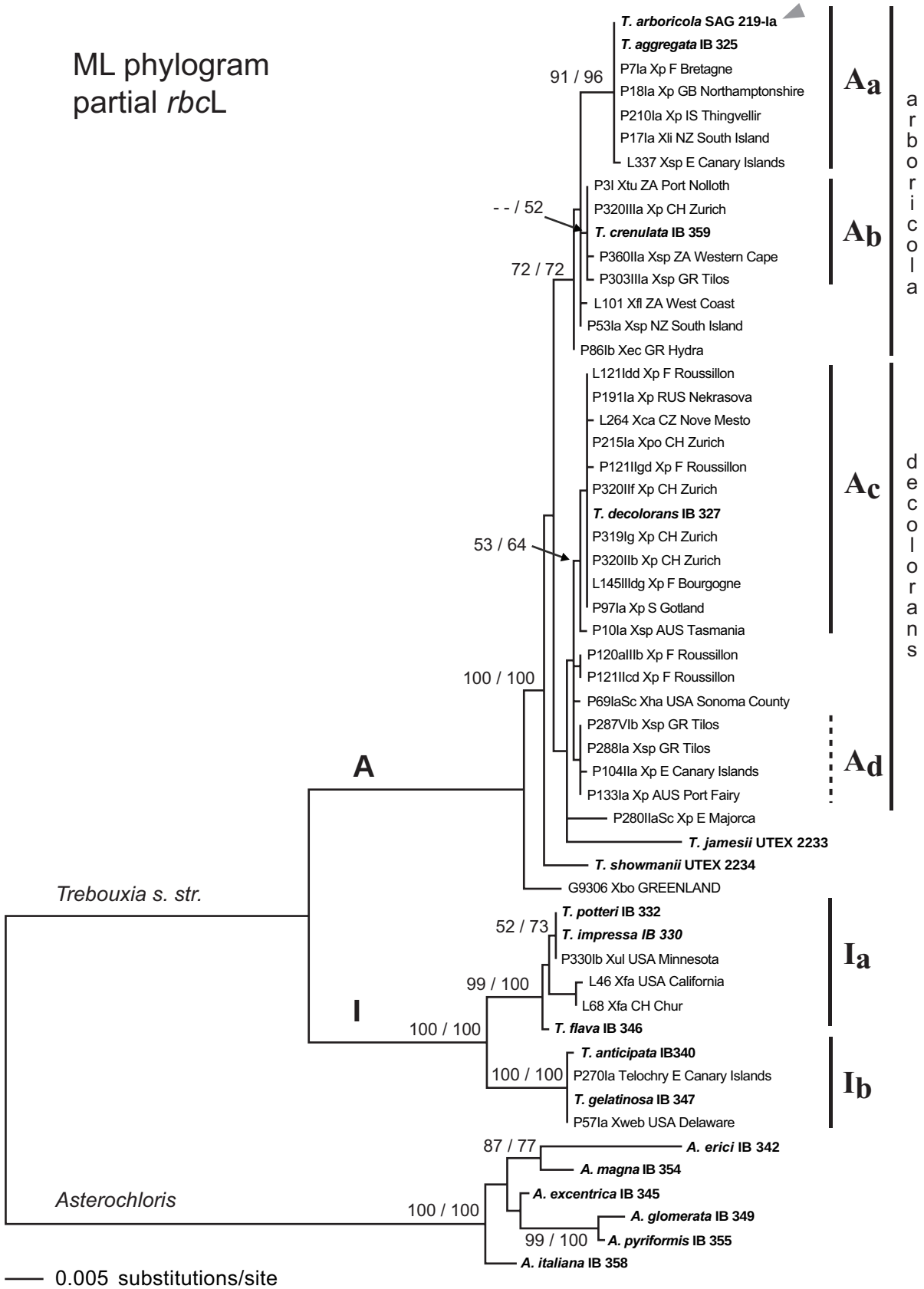
ML phylogram
partial rbcL
T. arboricola SAG 219-Ia
T. aggregata IB 325
P7Ia Xp F Bretagne
P18Ia Xp GB Northamptonshire
P210Ia Xp IS Thingvellir
P17Ia Xli NZ South Island
L337 Xsp E Canary Islands
P3I Xtu ZA Port Nolloth
P320IIIa Xp CH Zurich
T. crenulata IB 359
P360IIa Xsp ZA Western Cape
P303IIIa Xsp GR Tilos
L101 Xfl ZA West Coast
P53Ia Xsp NZ South Island
P86Ib Xec GR Hydra
L121Idd Xp F Roussillon
P191Ia Xp RUS Nekrasova
L264 Xca CZ Nove Mesto
P215Ia Xpo CH Zurich
P121IIgd Xp F Roussillon
P320IIf Xp CH Zurich
T. decolorans IB 327
P319Ig Xp CH Zurich
P320IIb Xp CH Zurich
L145IIIdg Xp F Bourgogne
P97Ia Xp S Gotland
P10Ia Xsp AUS Tasmania
P120aIIIb Xp F Roussillon
P121IIcd Xp F Roussillon
P69IaSc Xha USA Sonoma County
P287VIb Xsp GR Tilos
P288Ia Xsp GR Tilos
P104IIa Xp E Canary Islands
P133Ia Xp AUS Port Fairy
P280IIaSc Xp E Majorca
T. jamesii UTEX 2233
T. showmanii UTEX 2234
G9306 Xbo GREENLAND
T. potteri IB 332
T. impressa IB 330
P330Ib Xul USA Minnesota
L46 Xfa USA California
L68 Xfa CH Chur
T. flava IB 346
T. anticipata IB340
P270Ia Telochry E Canary Islands
T. gelatinosa IB 347
P57Ia Xweb USA Delaware
A. erici IB 342
A. magna IB 354
A. excentrica IB 345
A. glomerata IB 349
A. pyriformis IB 355
A. italiana IB 358
91 / 96
- - / 52
72 / 72
53 / 64
100 / 100
52 / 73
99 / 100
100 / 100
100 / 100
87 / 77
100 / 100
99 / 100
A
I
Aa
Ab
Ac
Ad
Ia
Ib
arboricola
decolorans
Trebouxia s. str.
Asterochloris
0.005 substitutions/site

Figure 2.2 ML phylogram of *rbc*L locus. Jackknife values calculated separately for 500 replicates by MP (first number) and NJ (second number) analyses are given at the nodes. *Asterochloris* sequences form the outgroup. Abbreviations used: *Xanthoria*: *Xbo: Xanthoria borealis, Xca: X. candelaria, Xec: X. ectaneoides, Xfl: X. flammea, Xli: X. ligulata, Xp: X. parietina, Xpo: X. polycarpa, Xtu: X. turbinata, Xsp:* unidentified *Xanthoria* or *Xanthomendoza sp. Xanthomendoza: Xfa: Xanthomendoza fallax, Xha: Xm. hasseana, Xul: Xm. ulophyllodes, Xweb: Xm weberi; Teloschistes: Telochry: Teloschistes chrysophthalmus.* A, C and I indicate *Trebouxia* clades as proposed by Helms (2003). P photobiont isolated, L whole lichen DNA used for amplification.

2.5 Discussion

2.5.1 Correlations among morphological and molecular data sets

A total of 124 ITS (101 used in analyses) and 54 *rbc*L sequences were newly generated in this study. These were compared with sequences obtained from databases and with unpublished ITS sequences of *Trebouxia* species and strains kindly provided by G. Helms.

A 28nt long insert, as found in *T. arboricola* (SAG 219-Ia), *T. aggregata* (UTEX 180), and *T. crenulata* (CCAP 219/2) (Helms *et al.* 2001), was also present in all newly generated sequences falling in subclades Aa and Ab. There was no difference in ITS tree topology after removing this insert. About 25% of nrITS sequences contained group I introns; these will be presented and discussed separately (Nyati, Bhattacharya & Honegger, in preparation).

The ITS sequence data obtained by Helms *et al.* (2001), Helms (2003) and in the present study indicate that *T. crenulata* and *T. aggregata* are conspecific with *T. arboricola*. Peršoh *et al.* (2004) consider *T. arboricola* synonymous with *T. decolorans*. As both are morphologically well distinguishable by the shape of their chloroplast (Friedl 1987) and cluster within different ITS and *rbc*L subclades both species names were retained in the present investigation. Some authors refer automatically to *T. arboricola* when ITS sequences fall into clade A *sensu* Helms (2003). Sooner or later morphospecies names given to taxa among the genera *Trebouxia* and *Asterochloris* will have to be revised, but large data sets comprising genetic and morphological data have to be accumulated to form a basis for future emendations.

Current phylospecies concepts in the genera *Trebouxia s. str.* and *Asterochloris* are based exclusively on the non-coding nrITS sequences, which are relatively short and highly variable. Until now only six SSU and seven LSU sequences are available for this group of

algae. Sequences of conserved regions such as *rbc*L, as achieved in the present study, gave additional insights in phylogenetic relations at species and supraspecific levels. Phylogenetic congruence was observed between data concerning the plastid *rbc*L loci and the nuclear ITS region (Tab. 2.4).

Table 2.4 Correlations of molecular markers and morphological characters in the genera *Trebouxia* and *Asterochloris* (Trebouxiophyceae, Chlorophyta)

Phylogeny			***Trebouxia*** *s.str* †	**Fine structure & Morphology** *sensu* Friedl (1989), modified				
ITS clade Helms (2003)	ITS clade present study	*rbc*L clade present study		arrangement of thylakoids	Pyrenoid type	chloroplast shape	cell shape	cell cycle
A7	A	n.d	*T. asymmetrica*	I	gi	6	ovoid	A
A9	A	n.d	*T. gigantea*	I	gi	6	ovoid	A
A8	A	n.d	*T. showmanii*	I	gi	6	ovoid	A
A10	A	n.d	*T. incrustata*	I	gi	6	ovoid	A
A2	Aa	Aa	*T. aggregata*	I	ar	3	globose	A
A2	Aa	Aa	*T. arboricola*	I	ar	3	globose	A
A2	Ab	Ab	*T. crenulata*	I	ar	4*	ovoid	A
A1	Ac	Ac	*T. decolorans*	I	ar	4*	globose	A
I1	Ia	Ia	*T. flava*	I	im	1	globose	B
I1	Ia	Ia	*T. impressa*	I	im	1	globose	A
A4	A	A	*T. jamesii*	I	im	2	globose	A
S3	S	n.d	*T. simplex*	I	im	2	globose	A
I1	Ia	Ia	*T. potteri*	I	im	5	globose	A
n.d	n.d	Ib	*T. anticipata*	I	ge	7	globose	B
I2	Ib	Ib	*T. gelatinosa*	I	ge	7	globose	B
C1**	C	n.d	*T. corticola*	I	co	9	globose	A
C2**	C	n.d	*T. galapagensis*	I	co	9	globose	A
C2**	C	n.d	*T. higginsiae*	I	co	9	globose	A
C1**	n.d	n.d	*T. usneae*	I	co	8	globose	B
Asterochloris (Tschermak-Woess) T. Friedl (ined.) (Rambold, Friedl & Beck 1998)								
n.d		outgroup	*A. magna*	I	ma	12	ovoid	B
n.d		outgroup	*A. excentrica*	II	ir	11	ovoid	B
n.d		outgroup	*A. glomerata*	II	ir	10	ovoid	B
n.d		n.d.	*A. irregularis*	II	ir	10	ovoid	B
n.d		outgroup	*A. italiana*	II	ir	10	ovoid	B
n.d		outgroup	*A. pyriformis*	II	ir	10	ovoid	B
n.d		outgroup	*A. erici*	II	er	10	ovoid	B

* chloroplast shape distinctly different *in T. crenulata* and *T. decolorans* (Gärtner 1985b). ** termed G in Helms (2003), now changed into C ("*corticola*"; Helms, personal communication)

Correlations of morphological data, as compiled by Friedl (1989b), and molecular data within the genera *Trebouxia* and *Asterochloris* are summarized in Table 2.4. ITS clade A, comprising most of the photobionts of Teloschistaceae investigated in this study, includes *Trebouxia* species from several morphological groupings. The morphology of the samples genetically identified in this study will have to be analysed in future investigations. Each of the ITS and *rbc*L subclades comprised numerous genotypes, many of

them being resolved with very low bootstrap support. It is interesting to see that identical ITS genotypes occurred in the same or even in different *Xanthoria* spp. from geographically different locations; examples are the photobionts of *X. parietina* from Corsica and of *X. capensis* from South Africa, of X. *parietina* from Otago (NZ) and from Cerdagne (F), of *X. parietina* from Nove Mesto (CZ), Zürich (CH) and Burgundy (F), or of *X. polycarpa* from Zürich, *X. parietina* from Zürich (CH), Gotland (S) and Burgundy (F). On the other hand *X. parietina* thalli collected side by side (populations 144 & 145 from Roussillon, SW France, 120 & 121 from Burgundy, France, and 319 & 320 from Zürich, (Switzerland) had partly the same, partly different ITS genotypes of mostly of the same subclade (Fig. 2.1).

2.5.2 Photobionts of the Teloschistaceae

All foliose and fruticose Teloschistaceae investigated in the present study associated with *Trebouxia* spp. belong either to ITS clade A or I *sensu* Helms (2003), or *rbc*L clades A or I, respectively. None of the samples had photobionts of ITS clades S or G *sensu* Helms (2003) or of the genus *Asterochloris*. All of the major clades comprise several morphospecies. A wide range of algal genotypes was found, comparable to the situation among photobionts of the genera *Letharia* (Kroken & Taylor 2000), *Cladonia* (Piercey-Normore 2004) or *Evernia* (Piercey-Normore 2006). As the focus was on the genus *Xanthoria* in general and on the *X. parietina* complex in particular the sample size for these groupings was distinctly larger than for the genera *Xanthomendoza* or *Teloschistes*. *X. candelaria* (CZ, IS) associated with *T. decolorans*, whereas Aoki *et al.* (1998), using microscopy techniques, identified *T. incrustata,* another representative from clade A *sensu* Helms (2003), from a sample collected in Antarctica.

Xanthomendoza spp. associated with photobionts of the *impressa* clade: *Xm. fallax* and *Xm. ulophyllodes* with *T. impressa* (subclade Ia), *Xm. novozelandica* and *Xm. weberii* with *T. gelatinosa* (sublade Ib). The other *Xanthomendoza* spp. had photobionts from clade A. *Xm. fulva* (Armenia) and *Xm. hasseana* (California) associated with *T. decolorans.* Two morphologically different thalli of *Xm. borealis*, a narrow- and a broad-lobed specimen collected at the same locality in Greenland, had different *T. decolorans* genotypes from different subclades (Fig. 2.1); their fungal partners turned out to not be conspecific (Eichenberger, unpubl.).

Due to very small sample sizes explored in the present study the range of compatible photobionts in representatives of the genera *Teloschistes* and *Josefpoeltia* remain unclear, *Trebouxia gelatinosa* (clade Ib) being the only green algal partner so far found associated with these taxa. *Fulgensia fulgida* was shown to associate with *T. asymmetrica* (Beck *et al.* 2002), a representative of ITS clade A. It is important to note that none of the foliose or fruticose species of Teloschistaceae examined in this investigation associated with photobionts from different clades. Until now only crustose taxa were found to associate with photobionts from different clades (Tibell & Beck 2001; Beck 2002; Helms 2003).

2.5.3 Photobionts of the genus *Xanthoria*

All *Xanthoria* species investigated in the present study had photobionts of ITS clade A *sensu* Helms (2003) (Fig. 2.1). No accurate geographic pattern can be seen in the present data set, but *T. decolorans* (subclades Ac and Ad) were almost exclusively found in corticolous samples, whereas *T. arboricola* occurred in saxicolous specimens (forming subclade Aa) in the northern and southern hemispheres and in many of the corticolous samples in the southern hemisphere (subclade Ab).

2.5.4 Photobionts of *Xanthoria parietina s. lat.*

Early investigators had already discovered a range of phenotypically different strains among *Trebouxia* isolates derived from thalli of *X. parietina*, which they interpreted as ecotypes (Thomas 1939; Warner 1954; Tomaselli 1956). Our present findings are in agreement with earlier reports, based on light and electron microscopic as well as molecular investigations, on *T. arboricola*, *T. decolorans* and *T. crenulata,* all members of ITS clade A *sensu* Helms (2003), being photobionts of *X. parietina* s. lat. (including *X. calcicola* and *X. ectaneoides*; Scherrer & Honegger 2003) (Ahmadjian 1960; Gärtner 1985b; Honegger & Peter 1994; Beck *et al.* 1998). Substrates are listed in Fig. 2.1. The *T. arboricola* photobiont of saxicolous *X. parietina* growing under a willow tree in Zürich was more closely related to the photobiont of a saxicolous *X. parietina* from Corsica than to the *T. decolorans* genotypes isolated from corticolous samples on the respective willow tree.

As pointed out by Rambold *et al.* (1998) and Peršoh *et al.* (2004) the Cladoniineae asso-

ciate with *Asterochloris* spp., whereas most foliose Lecanoriineae and Teloschistineae select *Trebouxia* spp. as photobiont. Nevertheless, there are some reports, based on microscopic investigations, on *Asterochloris* photobionts among Parmeliaceae (summarized by Rambold *et al.* 1998); these deserve re-investigation with molecular tools. *Asterochloris* spp. were reported twice as photobionts of *X. parietina. A. italiana,* originally isolated from an Italian sample as *Cystococcus Xanthoriae parietinae* (Tomaselli 1956) became the type strain of *A. italiana* (sub *Trebouxia italiana*), whose cells are mentioned to be multinucleate (Archibald 1975). Peršoh *et al.* (2004) speculate in this particular case on confusion of strains. Ahmadjian (2001) mentioned *A. irregularis* (sub *Trebouxia irregularis*) as photobiont of *X. parietina*, without giving any further details. He referred to *X. parietina* as an example of a lichen-forming fungus with a broad spectrum of acceptable photobionts. However, all genotypes of *X. parietina* examined in the present study (Eichenberger, unpubl.) associated with *Trebouxia* genotypes from the same ITS clade A. The few *Xanthoria parietina* and the phenotypically very similar, but phylogenetically different *Xanthoria* samples from Australia, Tasmania and New Zealand, all corticolous, had photobiont genotypes either from subclade Ab (*T. arboricola*), Ad (*T. decolorans*) or from the assembly of *T. decolorans* genotypes which fall between subclades Ac and Ad. Subclade Ad comprised an interesting assembly of genotypes isolated from corticolous samples growing in coastal areas from Brittany to Mallorca, Canary Islands, Greek Islands, South Africa and South Eastern Australia.

Photobiont diversity among *X. parietina* samples growing side by side in populations was examined. Our data set includes vouchers 120 and 121 collected on *Populus* and *Prunus* in South-Western France (Roussillon), vouchers 144 and 145 collected on *Salix* and *Viburnum* in Burgundy, and vouchers 319 and 320 from the university campus in Zürich, collected on *Parthenocissus* (319 I-IV) and *Salix* (320 I-II) or on sandstone underneath the *Salix* tree (320 III). All epiphytic specimens comprised *T. decolorans*, while the saxicolous specimen was associated with *T. arboricola*. With fingerprinting techniques (RAPD-PCR; data not presented) and ITS sequences (Fig. 2.1) several genotypes were resolved within the *T. decolorans* isolates from this particular site. Sterile-cultured isolates of the corresponding fungal partners were subjected to RAPD-PCR. They all turned out to be genetically heterogenous, but no major difference was noted between corticolous and saxicolous specimens (Honegger & Zippler, unpubl.).

2.5.5 Algal theft by *Xanthoria* spp. from adjacent *Physcia* species?

Based on the assumption of scarcity of free-living *Trebouxia* photobionts outside lichen thalli Ott (1987a, b; Ott *et al.* 2000) addressed the question how germinating ascospores of the always richly fertile *X. parietina* and *X. polycarpa,* both with no vegetative symbiotic propagules, acquire a compatible photobiont. She postulated temporary association of *Xanthoria* germlings with ultimately incompatible green algal cells and/or invasion of ascospore-derived germ tubes into the thalli of adjacent *Physcia* spp. (Lecanorineae, Lecanoromycetes), theft of their *Trebouxia* photobiont and subsequent development of a brightly yellow coloured thallus on or within the grey *Physcia* thalli. However, upon careful dissection presumed chimaerae of *X. parietina* and *Physcia tenella* and/or *P. adscendens* were invariably found to be juvenile thalli of *Xanthoria polycarpa* which, at young age, may be as grey as adjacent, small-lobed *Physcia adscendens* due to very low amounts of anthraquinones in their vegetative thallus, only pycnidial ostioles and apothecial discs being coloured by yellow anthraquinones (Honegger *et al.* 1996). In their inventory of photobiont diversity within crustose and foliose species of the *Physcietum adscendentis, X. parietina* being part of this community, Beck *et al.* (1998) showed with molecular markers that the photobionts of *Physcia* spp. are not associated with *X. parietina*. Extensive studies on photobiont diversity within the Physciaceae (Dahlkild *et al.* 2001; Helms 2003; Helms *et al.* 2001) support this view.

The present findings on photobiont diversity in *X. parietina and X. polycarpa* indicate that both species associate with photobionts of Clade ‘A’, i.e. with genotypes of *T. decolorans* (corticolous samples in Northern hemisphere) or *T. arboricola* (saxicolous *X. parietina* in Northern hemisphere, corticolous *X. polycarpa* in NZ). Thus photobionts of *Physcia tenella* and *P. adscendens* (subclade I1 sensu Helms 2003) are unlikely acceptable algal partners of either mycobiont. Sorediate structures, as described by Ott *et al.* (2000) as evidence for colonization of sorediate *Physcia* thalli by *X. polycarpa,* are within the range of phenotypic plasticity of the phylospecies *X. polycarpa* (Eichenberger, unpubl.). However, as already described by Ahmadjian (1960) with microscopy techniques and confirmed with molecular methods (Beck *et al.* 1998) *Buellia punctata*, an inconspicuous crustose species of the *Physcietum adscendentis*, has the same *T. decolorans* photobiont as *X. parietina* and, according to the present findings, as *X. polycarpa*.

Ahmadjian (2001; 2002) wrote about “lingering lichen myths” such as the belief that *Tre-*

bouxia spp. occur free-living outside lichen thalli and that they are photoautotrophic. Instead, he postulates *Trebouxia* spp. to be not independent organisms, but heterotrophic ones, “both in the lichen thallus and also growing independently in culture”. Our long-term culturing experiments on agarized non-nutrient mineral media leave no doubts about the ability of *Trebouxia* species to live as independent, photoautotrophic organisms. Based on diverse microscopic observations on free-living *Trebouxia* cells in nature (Tschermak-Woess 1978; Bubrick *et al.* 1984; Gärtner 1985a; Mukhtar *et al.* 1994; Ettl & Gärtner 1995; John *et al.* 2002; Rindi & Guiry 2003; Sanders 2005) it seems reasonable to assume *Trebouxia* species to be very widespread and distinctly more common than previously hypothesized. The fact that closely related *Trebouxia* genotypes occur in thalli of different sexually reproducing *Xanthoria* spp. with no vegetative propagules on different continents, as shown in the present investigation, indirectly indicates that these photobionts must be available in nature for re-lichenization events. Molecular probes might be used in future experiments to detect the availability of *Trebouxia* species in environmental samples.

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3 Novel group I LSU and SSU introns identified in lichen photobionts of the genera *Trebouxia* and *Asterochloris*

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3.1 Abstract

A novel group I intron was found at position 798 (*Escherichia coli* numbering) in the 26S rRNA gene of representatives of the genera *Trebouxia* de Puymaly and *Asterochloris* (Tscherm.-Woess) T. Friedl *ined*. These are green algal photobionts of Lecanoromycetes (lichen-forming ascomycetes). The focus of this study is *Trebouxia* species associated with Teloschistaceae. The 798 intron was found in about 25% of photobionts that were studied including several reference strains obtained from algal culture collections. An alignment of 26S rDNA intron sequences revealed high similarity of these sequences and the presence of peculiar P, Q, R, and S catalytic core sequences within the intron. The 798 group I intron phylogeny was largely congruent with the ITS phylogeny, indicating that the insertion of the 798 intron most likely occurred in the common ancestors of the genera *Trebouxia* and *Asterochloris.* The intron was vertically inherited in some taxa, but lost in others. The high sequence imilarity of this intron to one found in *Chlorella ellipsoidea* suggests that the 798 intron was either present in the common ancestor of Trebouxiophyceae, or that its present distribution results from horizontal transfers, followed by vertical inheritance and loss. Our present data regarding the SSU 1512 group I intron within the genera *Trebouxia* and *Asterochloris* support published hypotheses on repeated lateral transfers of this intron among some, but loss within other lineages. An atypically long 1512 intron sequence was found in the SSU rDNA of a *Trebouxia arboricola* isolate from a South African sample of *Xanthoria turbinata.* The intron was 460 base pairs (bp) in length with an insertion of 1090 bp at a conserved site.

3.1.1 Key words:

LSU 798 group I intron, SSU 1512 group I intron, Trebouxiophyceae

3.2 Introduction

Group I introns occur in different genes from a variety of organisms (Michel and Westhof 1990). As of June 2004, about 1400 group I introns have been reported from eukaryotic genomes (Haugen, Simon and Bhattacharya 2005). Of these, 800 are found in the nucleus at 47 different sites in SSU rDNA and 44 sites in LSU rDNA, 220 occur in mitochondrial genes and 370 in plastid DNA. Group I introns are characterized by conserved core sequences (P, Q, R and S) and conserved secondary structures. Interestingly, group I introns are always located at highly conserved regions of rRNA genes (Johansen, Muscarella and Vogt 1996). All group I introns catalyze excision from primary transcripts using guanosine as a cofactor. Many of these ribozymes are autocatalytic, whereas others rely on host derived factors to facilitate splicing (Cech 1988; Cech, Damberger and Gutell 1994). Five major subgroups of group I introns, termed IA, IB, IC, ID, and IE are recognized based on secondary structure (Michel and Westhof 1990).

In eukaryotic microorganisms, horizontal transfer of group I introns appears to be the rule, rather than the exception. Therefore, the mapping and phylogenetic characterization of the widespread, but scattered nuclear group I introns clarifies potential pathways and mechanisms of intron movement among eukaryotes (Einvik, Elde and Johansen 1998). Various studies have focused on the mechanisms underlying group I intron mobility. Many group I introns in the genomes of plastids, mitochondria, phages and eubacteria contain open reading frames (ORFs), that encode endonucleases (ENases). These ENases mediate the sequence-specific ‘homing’ of group I introns into allelic sites (Dujon 1989). Intron movement can also occur via reverse splicing, as demonstrated for the *Tetrahymena thermophila* LSU group I intron.
Intron loss appears to be common as demonstrated by the ‘optional’ distribution of group I introns within closely related taxa (Bhattacharya et al. 1996; Bhat-

tacharya, Friedl and Damberger 1996; Mourier and Jeffares 2003). At the RNA level, intron loss seems to occur by reverse transcription of an intron-less RNA followed by general recombination with the intron containing genomic copy of the coding region (Dujon 1989). In crown group taxa (e.g., green algae, charophytes, red algae, ciliates, fungi) putatively active open reading frames (ORFs) have not yet been found in group I introns; thus their intron mobility seems to be mediated by reverse splicing and loss (Bhattacharya, Friedl and Damberger 1996).

The lichen symbiosis is particularly interesting with regard to group I intron presence and distribution. Lichen-forming ascomycetes contain several group I introns of different sizes in the SSU rDNA, many of them being inserted at unique positions. The SSU rDNA intron variation observed by Depriest (1993a) in a population of *Cladonia chlorophaea* was due to optional group I introns, which varied in number, position, restriction pattern and size. Some of these insertion positions are unique, whereas others (516, 943, 1046, & 1506) were also found in other organisms including unicellular green algae (Gargas, Depriest and Taylor 1995). However, no direct evidence has yet been found demonstrating SSU rDNA group I intron movement between the fungal and algal partners in the lichen symbiosis.

Because the green algal photobionts of lichen-forming fungi contain numerous nuclear-encoded rDNA group I introns, they are a model group to study the origin and phylogeny of these sequences (Bhattacharya et al. 1994; Bhattacharya et al. 1996; Bhattacharya, Friedl and Damberger 1996; Bhattacharya et al. 1998). Friedl et al. (2000) concluded that the SSU rDNA 1512 group I intron was present in the common ancestor of the green algal classes Trebouxiophyceae, Chlorophyceae and Ulvophyceae and that it was laterally transferred at least three times among *Trebouxia* spp. They also concluded that intron loss was a common event during chlorophyte evolution. The green algal SSU group I introns at insertion sites 1056, 1506, and 1512 (position relative to the *E. coli* coding region) form distinct phylogenetic lineages based on insertion site (Bhattacharya et al. 1994; Bhattacharya, Friedl and Damberger 1996). The intron phylogenies were largely congruent with the rDNA phylogeny (host cell

phylogeny), suggesting vertical inheritance of the introns rather than lateral transfers during the evolution of green algae.

In a study of genetic diversity among the green algal photobionts of Teloschistaceae (Lecanoromycetes, lichen-forming ascomycetes) numerous *Trebouxia* strains and species were found to contain group I introns (Nyati, Scherrer and Honegger, unpubl.). The aims of the present study are, 1) to characterize a newly found LSU group I intron at site 798; 2) to determine the distribution of the 798 and SSU 1512 group I introns in this group of organisms; 3) to characterize an exceptionally long SSU 1512 group I intron in a *Trebouxia arboricola* isolate from *Xanthoria turbinata*; and 4) to investigate correlations between nrITS and intron phylogenies among green algal lichen photobionts of the genera *Trebouxia* and *Asterochloris*.

3.3 Materials and Methods:

3.3.1 Lichen collection, storage and photobiont isolation and culture

Several *Xanthoria spp*. Including the broadly sampled *X. parietina* were systematically or punctually collected (Table3.1). The lichens were stored and the photobionts isolated and cultured as described by Honegger (2003, 2004). *Trebouxia* and *Asterochloris* type strains were obtained from Culture Collection of Algae at the University of Innsbruck (IB/ASIB), Austria for comparison (Table 3.2)..

Table 3.1 List of samples analyzed in this study for LSU and SSU introns

Trebouxia species †	Isolated from *Xanthoria* sp.	Country	Voucher No*	ITS accession no.	LSU accession no.
T. arboricola	*X. calcicola*	CH	P-105-I-a	AJ969542	AM261263
T. arboricola	*X. calcicola*	CH	P-141-II	AJ969552	
T. arboricola	*X. capensis*	ZA	P-306-I-a	AJ969591	AM261277
T. arboricola	*X. ectaneoides*	F	P-158-IV-mc	AJ969560	
T. arboricola	*X. ectaneoides*	I	L-43	AJ969523	
T. arboricola	*X. ectaneoides*	TN	P-174-II-aA	AJ969565	
T. arboricola	*X. ligulata*	NZ	P-17-I-a ‡	AJ969518	AM261260
T. arboricola	*X. ligulata*	NZ	P-17-II-a	AJ969519	
T. arboricola	*X. ligulata*	NZ	P-53-I-a	AJ969528	
T. arboricola	*X. ligulata*	NZ	P-54-II-a	AJ969530	
T. arboricola	*X. parietina*	GB	P-18-I-a	AJ969520	
T. arboricola	*X. parietina*	CY	P-5-I-a-A	AJ969510	
T. arboricola	*X. parietina*	IS	P-198-II-a	AJ969568	
T. arboricola	*X. parietina*	IS	P-210-I-a	AJ969570	
T. arboricola	*X. parietina*	F	P-7-I-a	AJ969512	
T. arboricola	*X. parietina*	CH	P-320-III-a	AJ969604	AM261279
T. arboricola	*X. polycarpa*	NZ	P-48-III-a	AJ969526	
T. arboricola	*X. sp.*	CY	P-5-I-a	AJ969510	
T. arboricola	*X. sp.*	AUS	P-276-I-a ‡	AJ969581	AM261275
T. arboricola	*X. sp.*	ZA	P-360-II-a ‡	AJ969609	AM261281
T. arboricola	*X. turbinata*	ZA	P-3-I §	AJ969509	
T. decolorans	*X. candelaria*	IS	P-205-II-a ‡	AJ969569	AM261272
T. decolorans	*X. hasseana*	USA	P-400-Ia	AM159210	
T. decolorans	*X. parietina*	AUS	P-10-I-a ‡	AJ969515	AM261258
T. decolorans	*X. parietina*	AUS	P-11-Ia	---	AM261259
T. decolorans	*X. parietina*	AUS	L-275-II ‡	AJ969580	AM261274
T. decolorans	*X. parietina*	USA	L-8 ‡	AJ969513	AM261256
T. decolorans	*X. parietina*	USA	L-9 ‡	AJ969514	AM261257
T. decolorans	*X. parietina*	S	P-97-I-a	AJ969539	
T. decolorans	*X. parietina*	F	P-121a-IIIb	AJ969545	AM261264
T. decolorans	*X. parietina*	F	P-121-I-b	AJ969549	AM261266
T. decolorans	*X. parietina*	F	L-121-I-d	AJ969546	AM261265
T. decolorans	*X. parietina*	F	P-121-II-c	AJ969550	AM261267
T. decolorans	*X. parietina*	F	P-121-II-g	---	AM261268

T. decolorans	*X. parietina*	F	P-144-III-b	AJ969554	
T. decolorans	*X. parietina*	F	P-144-III-h	AJ969556	
T. decolorans	*X. parietina*	F	L-145-III-g	AJ969557	AM261270
T. decolorans	*X. parietina*	F	P-145-I-f-B	AJ969558	AM261271
T. decolorans	*X. parietina*	E	P-280-IIa	AJ969583	AM261276
T. decolorans	*X. parietina*	CH	P-319-I-g ‡	AJ970889	AM261278
T. decolorans	*X. parietina*	CH	P-319-II-a	AJ969595	
T. decolorans	*X. parietina*	CH	P-320-I-d2	AJ969599	
T. decolorans	*X. parietina*	CH	P-320-II-b	AJ969600	
T. decolorans	*X. parietina*	CH	P-320-II-f	AJ969603	
T. decolorans	*X. polycarpa*	USA	P-71-II-b	AJ969535	AM261262
T. decolorans	*X. polycarpa*	CH	P-215-I-a	AJ969572	
T. gelatinosa	*Xanthomendoza weberi*	USA	P-57-I-a	AJ969532	AM261261

†*Trebouxia* species identified according to ITS and *rbc*L phylogenetic analysis (Nyati, Scherrer, and Honegger, unpubl.); * P indicates photobiont isolated, L indicates whole lichen DNA used for PCR amplification; ‡ both introns present; § *T. arboricola* isolated from *X. turbinata* which had an insert of 1090 bases within 1512 group I intron.

Table 3.2 ITS and partial LSU accession numbers of reference algal strains used in this study

Algal species	Strain *	LSU accession No.	ITS accession No.	Reference †
Asterochloris erici	UTEX 912		AF345441	Piercey-Normore et al. (2001)
A. erici	IB 342	AM261248		This study
A. excentrica	IB 345	AM261249		This study
A. glomerata	UTEX 897		AF345405	Piercey-Normore et al. (2001)
A. glomerata	IB 349	AM261252		This study
A. italiana	IB 358	AM261253		This study
Chlorella ellipsoidea	C-87	D17180		Aimi et al. (1994)
Trebouxia anticipata	IB 340	AM261247		This study
T. arboricola	92.011A1		AJ249481	Friedl et al. (2000)
T. arboricola	92.011C3		Z68703	Bhattacharya et al. (1996)
T. arboricola ‡	SAG 219-Ia		Z 68705	Bhattacharya et al. (1996)
T. asymmetrica	B207		AF344177	Beck et al. (2002)
T. corticola	UTEX 909		AJ249566	Friedl et al. (2000)
T. decolorans	IB 327	AM261243	Unpubl.	Unpublished
T. galapagensis	UTEX 2230		AJ249567	Friedl et al. (2000)
T. gelatinosa	87.072B1		AJ249575	Friedl et al. (2000)
T. higginsiae	UTEX 2232		AJ249574	Friedl et al. (2000)
T. impressa	87.017E1		AJ249570	Friedl et al. (2000)
T. showmanii	IB 337	AM261246	AF242470	Kroken et al. (2000)

IB: Culture collection of algae at the University of Innsbruck; SAG: Algal culture collection, University of Göttingen; UTEX: algal culture collection, University of Texas. All LSU sequences generated in this study; unpublished ITS sequence kindly provided by T. Friedl and G. Helms Type strain of the genus *Trebouxia*

3.3.2 DNA extraction, PCR amplification and sequencing

Genomic DNA from algal isolates or whole lichens was extracted using the GFX PCR, DNA and Gel Band Purification Kit (Amersham Biosciences, Little Chalfont) according to the manufacturer's protocol. PCR amplification of a partial LSU rDNA fragment (position 660-1100 of *T. asymmetrica,* Z95380) was done with newly designed primers (Table 3.3). The PCR reactions were performed in 50 µl reaction volume containing a reaction mix of 0.2mM of each of four dNTPs, 3µl of each PCR primer (10µM), and 1.25 U of Taq DNA polymerase (SIGMA) in 1X PCR buffer provided with the polymerase with the following conditions: initial denaturation for 3' at 95°C, followed by 30 cycles of (30" at 95°C, 30" at 60°C, 1' at 72°C) and final extension for 10' at 72°C. Primers were newly designed for amplification of 1512 introns and adjoining SSU rDNA region (Table 3.3). PCR amplification was carried out with the following settings: 3' at 95°C, 30 cycles of 30" at 95°C, 40" at 56°C, and 1'20" at 72°C with final extension at 72°C for 10'. Sequencing was carried out using ABI Prism BigDye Terminator Mix V3.0 Cycle Sequencing Kit following the protocol of manufacturer and analyzed on an Applied BioSystem ABI 3730 DNA Analyzer.

Table 3.3 List of newly designed primers used in this study

Primer	Target	Sequence (5'–––>3')
Intron 1 (fwd)	SSU intron	CTGTCACTAGACTGAGTGC
Intron 2 (rev)	SSU intron	CCAGTTTAGAGGCTCGAATC
Intron 3 (rev)	SSU intron	GGWCCGACTATATCTTAAGC
Intron 4 (fwd)	SSU intron	TTGTTGTAAAGGGCTCCACT
Intron 5 (rev)	SSU intron	ACTCCTGTAAGCTCTCCTTCC
LSU fwd	LSU	TTTAACACCCATGAGACGCAAGTAAC
LSU rev	LSU	GCCTTAACTCAGCTTTCGGTTCA

3.3.3 Sequence alignment and phylogenetic analysis

Sequence contigs were assembled using Sequencer™ 4.2.2 (Gene Codes Corp. Ann Arbor, USA.) and ambiguous positions were manually corrected. Clean sequences were aligned under ClustalX (Thompson et al. 1997). The resulting alignment was visually checked for any discrepancies and manually corrected on MacClade V5.0 (Maddison and Maddison 2002). Intron insertion position was calculated based on BLAST searches and sequence alignments. Maximum Likelihood (ML), Maximum parsimony (MP) and Neighbour joining (NJ) analyses were carried out using PAUP 4.0 b10 (Swofford 1998). The host cell phylogeny, as presented in Figures 3.1A and C, is based on alignments of the complete ITS region, including the 5.8S rRNA gene.

3.3.4 PCR test to confirm presence of 1512 group I introns in photobiont isolates

Intron specific primers were used for PCR amplification reactions, which were carried out with genomic DNA isolated from algal strains and from whole lichen DNA extracts. Several different primer combinations were tested to exclude any PCR bias. These tests were performed to calculate absolute frequencies of introns in investigated algal isolates.

3.4 Results and Discussion

3.4.1 *Trebouxia* LSU 798 group I intron

Prior to this study the 26S rRNA gene was poorly investigated in green algal lichen photobionts of the genera *Trebouxia* and *Asterochloris* with only 7 LSU rDNA sequences having been published thus far (Friedl and Rokitta 1997; Tab. 3.2). LSU rDNA introns had not been found in these taxa. In the current investigation 28 new LSU rDNA group I introns were found, which are all inserted at position 798 and belong to the group IB introns. In a secondary structure based alignment of *Trebouxia* LSU introns the sequence of *Chlorella ellipsoidea* C-87 was included (Aimi et al. 1994) because this intron was also 445 base pairs (bp) in length and had all the characteristic RNA foldings (P1-P10) typical of group IB introns. The 5'- and 3'- splicing sites are between U and A, and bet-

ween G and C, respectively. The conserved nature of the P1 and P10 loops indicates that reverse transcription might be possible and that this intron could move to new homologous sites. The adjoining exon regions were highly conserved.

The LSU 798 group I intron phylogeny (Fig. 3.1 B) was congruent with the ITS host cell phylogeny (Fig. 3.1 A), indicating vertical inheritance of the intron. The only exceptions are the introns in *T. showmanii* and *T. decolorans* isolated from *X. parietina* (P-280-IIa) (group 5 in Figs. 3.1 A and B), which might have been laterally transferred. A comparison of the conserved core catalytic sequences (P, Q, R, S) revealed very high sequence similarity with a very clear pattern in nucleotide substitution between *Trebouxia* subclades and the *Asterochloris* clade (Fig. 3.2). Due to the conserved secondary structures among the LSU group I intron of representatives of the genera *Trebouxia* and *Asterochloris* (Microthamniales) and of *Chlorella ellipsoidea* (Chlorellales) we hypothesize that this intron was present in the common ancestor of the genera *Trebouxia* and *Asterochloris* and perhaps in the common ancestor of the Microthamniales and Chlorellales within the Trebouxiophyceae.

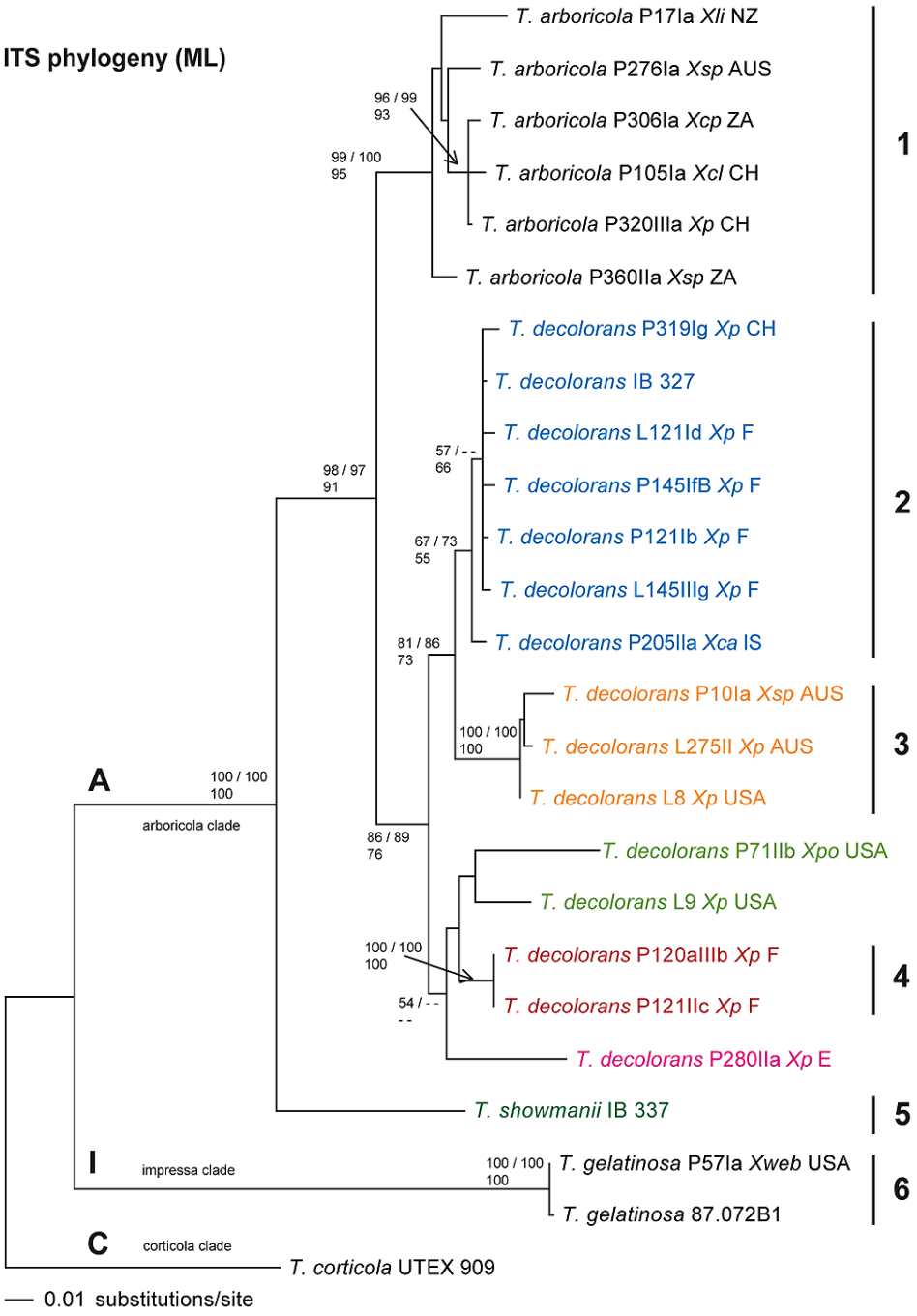

Figure 3.1 A Phylogeny of *Trebouxia* s. str. nuclear ribosomal internal transcribed spacer region (complete ITS region, including the 5.8S rRNA gene) for 798 group I LSU intron as inferred using Maximum Likelihood (ML) analysis. Jackknife values given were calculated separately for 500 replicates of a Maximum Parsimony (MP; first row, first number), and a Neighbor Joining analysis (NJ; first row, second number) and 100 resamplings of a Maximum Likelihood (ML; second row) analysis and indicated at the corresponding branches. Sequences obtained from database are indicated in table 3. Photobiont identification was done on the basis of ITS and *rbc*L phylogenetic analyses (Nyati, Scherrer and Honegger submitted) Clades A, I and C represent 'arboricola', 'impressa' and 'corticola' respectively as proposed by Helms (2003). *Asterochloris* ITS sequences not used in analyses due to very high sequence variation. Abbreviations used: *Xca: Xanthoria candelaria, Xcl: X. calcicola, Xcp: X. capansis*, *Xec: X. ectaneoides, Xli: X. ligulata, Xp: X. parietina, Xpo: X. polycarpa, Xtu: X. turbinata, Xsp: Xanthoria sp., Xweb: Xanthomendoza weberi.*

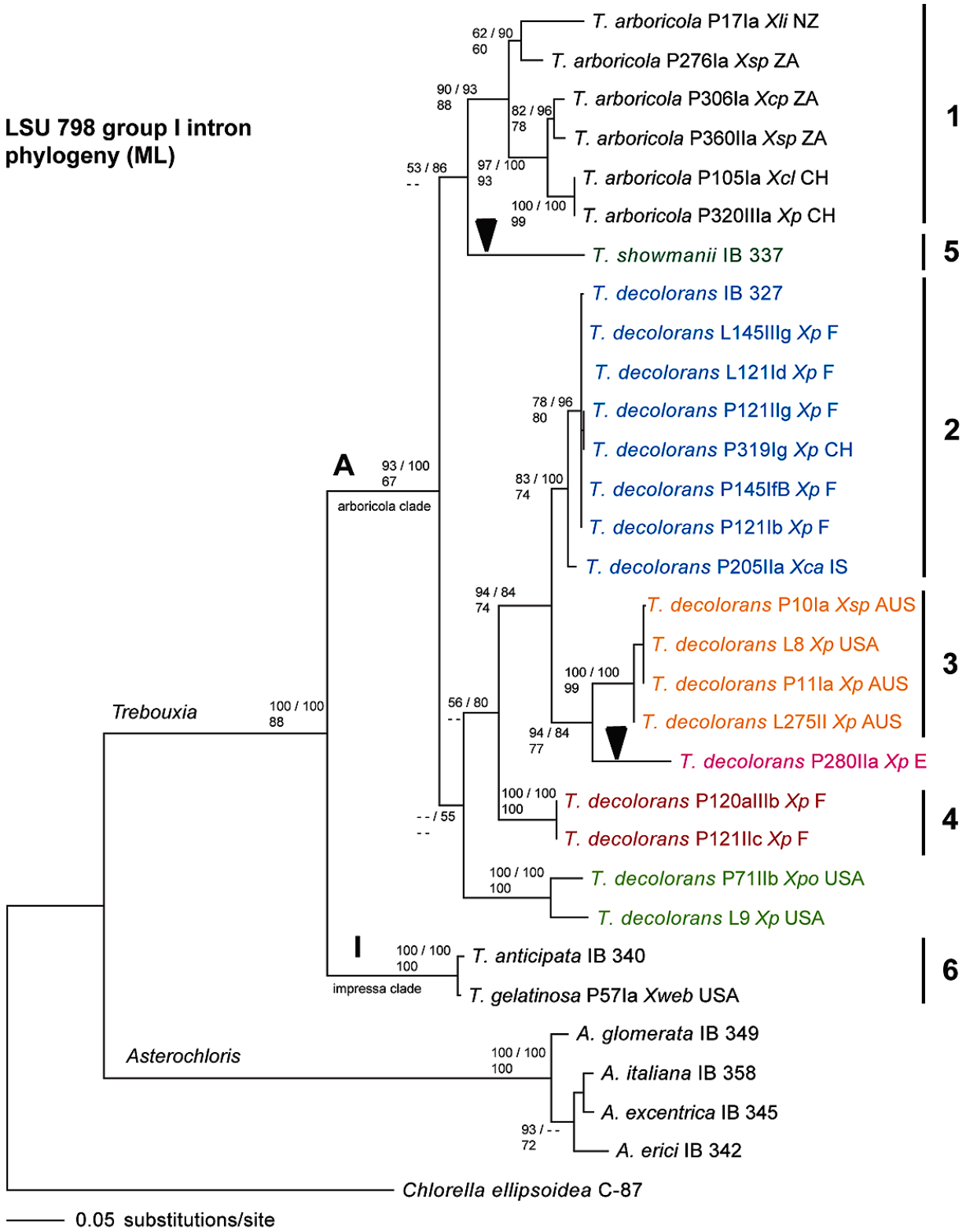

Figure 3.1 B Single most likely phylogram resulting from Maximum Likelihood (ML) analysis of the LSU 798 group I intron in representatives of *Trebouxia* s. str. and *Asterochloris* Tsch-Woess. Jackknife values were calculated separately for 500 replicates of a MP (first row, first number) and NJ (first row, second number) and 100 replicates of a ML analysis (second line) and indicated at the corresponding branches. Intron sequences of *Chlorella ellipsoidea* strain C-87 was obtained from database and used as outgroup taxa. Arrowhead indicates potential site for intron lateral transfer.

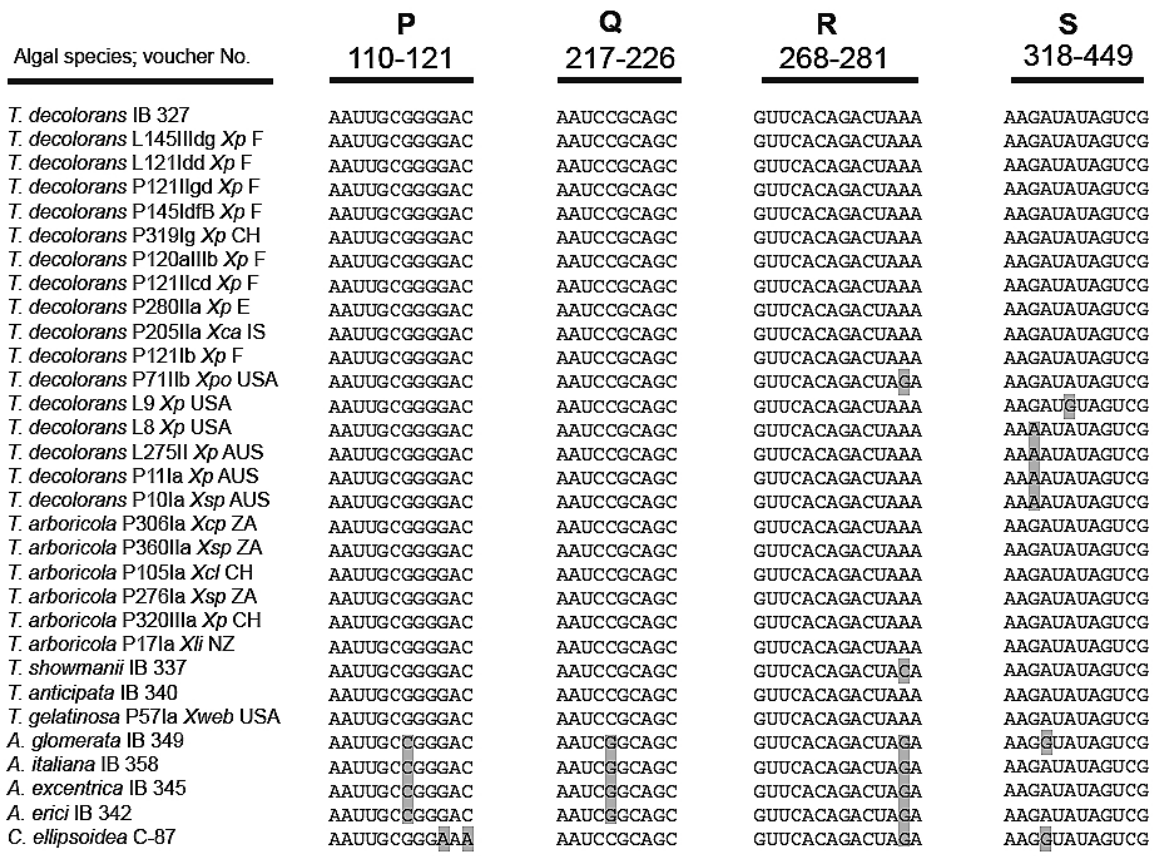

Figure 3.2 Comparison of catalytic core (PQRS) sequences of LSU 798 group I introns in representatives of the genera *Trebouxia* s. str. and *Asterochloris*. Type strains were obtained from the culture collection of algae at the University of Innsbruck, Austria (IB), whereas photobionts of investigated *Xanthoria* and *Xanthomendoza* species were identified on the basis on ITS and *rbc*L analyses. The positions of P, Q, R, S regions are based on the reference sequence of the *Chlorella ellipsoidea* strain C-87 intron (accession number D17180). Nucleotides in the shaded box indicate differences with regard to the reference sequence of *Trebouxia decolorans* (IB 327). Abbreviations used: *Xca: Xanthoria candelaria, Xcl: X. calcicola, Xcp: X. capensis*, *Xec: X. ectaneoides, Xli: X. ligulata, Xp: X. parietina, Xpo: X. polycarpa, Xtu: X. turbinata, Xsp: Xanthoria sp., Xweb: Xanthomendoza weberi*

3.4.2 *Trebouxia* 1512 group I intron

In an analysis of the genetic diversity among *Trebouxia* photobionts of Teloschistaceae, 124 ITS sequences were generated, 32 of them containing a group I intron (Nyati, Scherrer and Honegger, unpubl.). These intron harboring *Trebouxia* photobionts belong to the “arboricola” clade A as described by Helms (2003). In all except one photobiont sample the insert was between 434

bp and 530 bp in length. The 460 bp intron of the *T. arboricola* photobiont of the African *Xanthoria turbinata* (accession No. AJ969509) had an additional insertion of 1090 bp at a conserved site (P8A), making the entire insert 1550 bp in length. Although this intron is very long, it does not appear to encode an ORF for a homing endonuclease. No similarities were found between this longer intron and complex nested inserts found in 18S rDNA of ascomycetes, where putative spliceosomal introns were inserted within group I introns (Depriest and Been 1992; Myllys, Kallersjo, and Tehler 1999; DePriest 2004).

In comparison with published data (Bhattacharya, Friedl, and Damberger 1996) all inserts at position 1512 were identified as group I introns. Twenty nine newly generated intron sequences together with already published data, were analyzed using phylogenetic methods (Fig. 3.1 D) and compared with the ITS phylogeny (Fig. 3.1 C) to detect potential lateral transfers (Bhattacharya, Friedl and Damberger 1996; Friedl et al. 2000). The ITS and SSU 1512 group I intron phylogenies were generally congruent (Fig. 3.1 C, D), indicating overall vertical inheritance. However, three interesting incongruities were found (marked with arrowheads in Fig. 3.1 D): 1) the intron sequence of *Trebouxia corticola* (UTEX 909, a free-living alga from tree bark in Webster, MA, USA isolated by V. Ahmadjian in 1959), a representative of the “corticola” clade C according to Helms (2003), falls near three intron sequences (marked as group 6 in Figs. 3.1 C and D) from clade A. These *Trebouxia decolorans* photobionts were identified from *X. parietina* thalli from Barossa valley (AUS) and Sonoma county (California, USA) and from an unnamed *Xanthoria* species from Tasmania (AUS). 2) the intron sequence of *Trebouxia impressa* (Friedl et al. 2000), a representative of the “impressa” clade I according to Helms (2003), falls near two *T. decolorans* intron sequences (marked as group 5 in Figs. 3.1 C and D) from clade A. These *Trebouxia decolorans* photobionts were found in thalli of *Xanthoria hasseana* and *X. parietina* in California. 3) intron sequences of *T. arboricola* isolates from *X. ectaneoides* (Sicily, Italy), *X. parietina* (Avenches, Switzerland), *X. polycarpa* (Otago, New Zealand) and *X. turbinata* (Port Nolloth, South Africa) formed a separate group outside the ‘decolorans clade’ (clade 7), with 100% jackknife support in all three analyses (Fig. 3.1D). All these incongruities might have resulted from lateral intron transfers

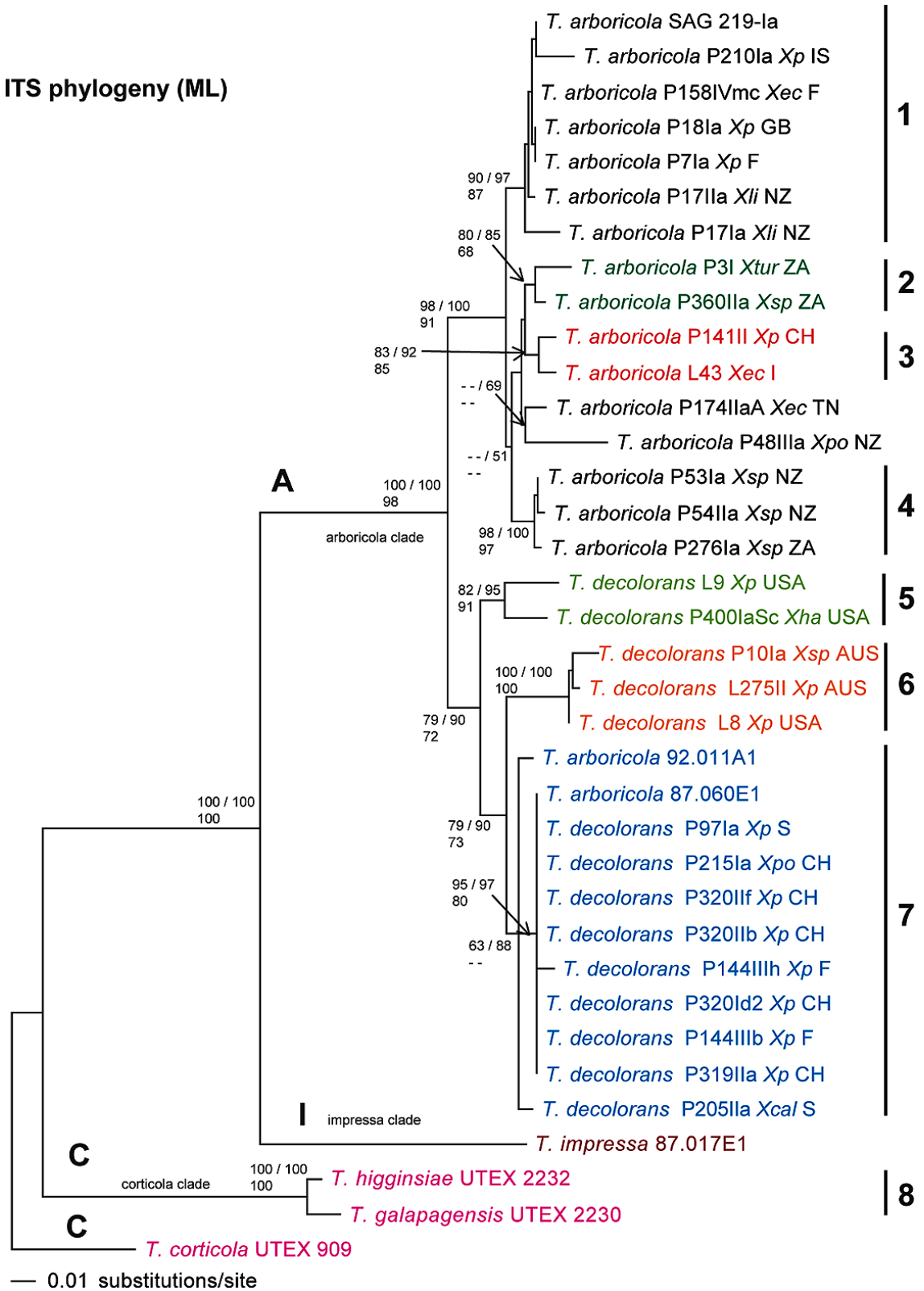

Figure 3.1 C Maximum Likelihood (ML) phylogeny of the complete ITS region, including the 5.8S rRNA gene, in representatives of *Trebouxia* s. str. for investigating any potential lateral transfer of SSU 1512 group I intron. Jackknife values calculated separately for 500 replicates of a MP (first row, first number) and NJ (first row, second number) and 100 replicates of a ML analysis (second row) and indicated at corresponding branches. *Trebouxia arboricola* 92.011A1 and 87.060E1 isolated from Physciaceae have been identified by Friedl et al. (2000) on the basis of ITS sequence analysis.

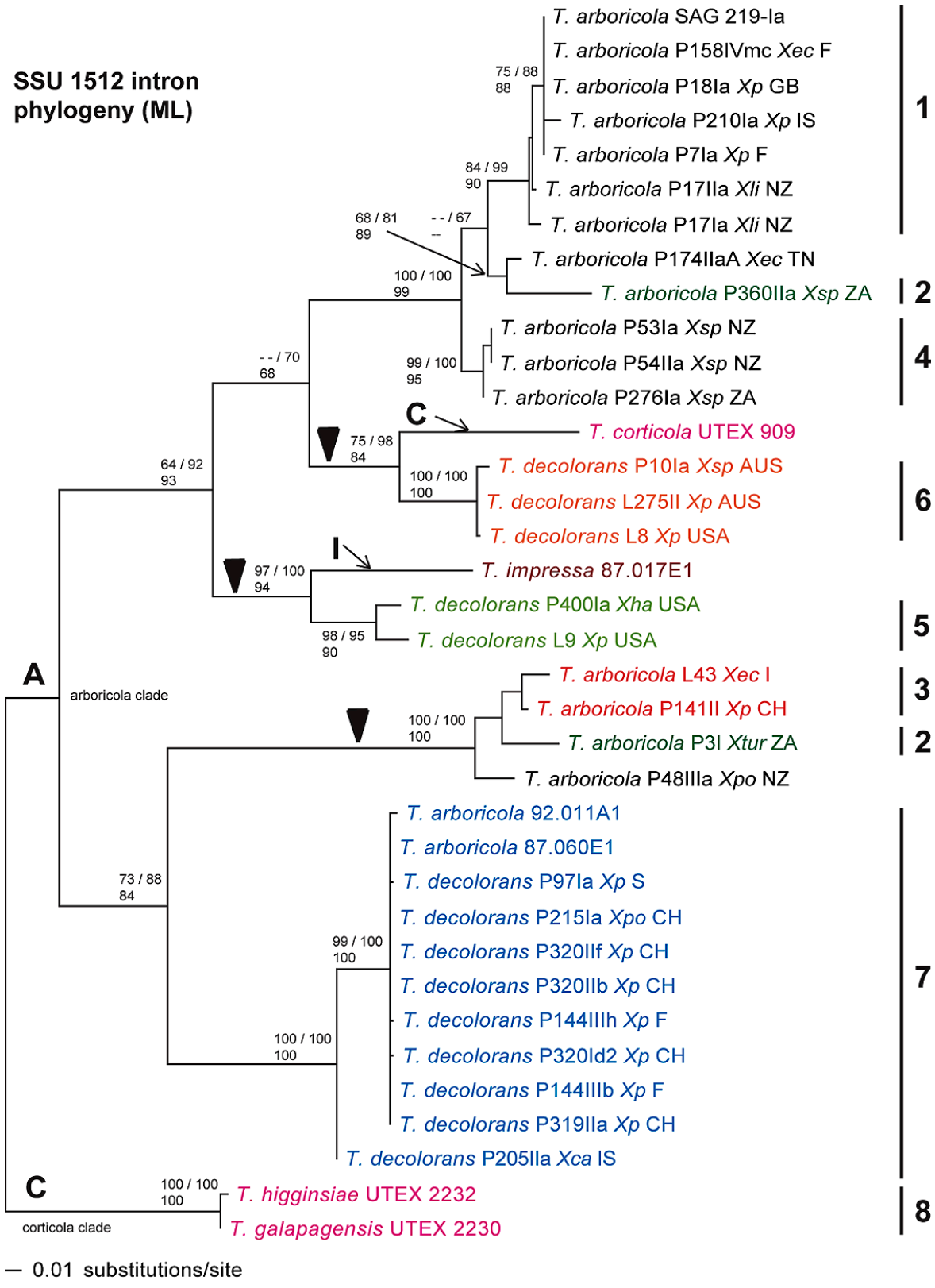

Figure 3.1 D Maximum Likelihood phylogram of 1512 group I intron sequences. Jackknife values were calculated separately for 500 replicates of a MP (first row, first number) and NJ (first row, second number) and 100 replicates of a ML analysis (second row) and indicated at corresponding branches. Arrowheads indicate potential sites for intron lateral transfers.

3.4.3 LSU and SSU group I intron distribution in *Trebouxia* and *Asterochloris* spp.

Introns in both SSU and LSU rDNA were found in only 9 *Trebouxia* isolates. The distribution of these taxa in the host tree (Fig. 3.1A and C) and in the photobionts of Teloschistaceae (Tab. 3.1) follows neither a geographic nor a taxonomic pattern. *Trebouxia* isolates from thalli of *Xanthoria parietina* from the Northern Hemisphere are however over-represented in this analysis, and only one sample was studied from some of the other species. In a survey of the genetic diversity among *Trebouxia* photobionts of Teloschistaceae (n = 124) the SSU 1512 group I intron was found in 50% of *T. arboricola*, but in only 23 % of *T. decolorans* samples (Nyati, Scherrer and Honegger, unpubl.). Friedl et al. (2000) found a SSU 1512 intron in 28% of 85 algal strains that were tested. In a PCR based screening of sterile cultured isolates of *T. arboricola* or *T. decolorans*, respectively, from populations of *Xanthoria parietina* and *X. ectaneoides* from maritime, coastal, rural and urban sites a very unequal distribution of SSU 1512 group I introns was observed, ranging from none to 87% (Tab. 3.4). This most likely reflects the highly dynamic nature of intron gain and loss. The *Trebouxia* photobionts of *Xanthoria ectaneoides* or *X. parietina* populations with the highest and lowest percentages of SSU 1512 group I introns were collected less than 1 km apart from each other. The first was found on granite rock in the supralittoral fringe of a tiny island (Île verte), the other on shrubs in the old port of Roscoff in Brittany (NW France; Tab. 3.4).

Table 3.4 Presence of SSU1512 group I intron in axenically cultured *Trebouxia* isolates from five *Xanthoria* populations collected in Europe

Photobiont	Voucher No.	Mycobiont	Collecting site	Substrate	Intron*/ isolates, %
Trebouxia arboricola	158	*X. ectaneoides*	maritime, F	saxicolous	20/23, 87%
T. decolorans	120-121	*X. parietina*	rural, F	epiphytic	1/25, 4%
T. decolorans	144-145	*X. parietina*	rural, F	epiphytic	6/63, 9.5%
T. decolorans	164	*X. parietina*	coastal, F	epiphytic	0/12, 0%
T. decolorans, T. arboricola	319-320	*X. parietina*	urban, CH	epiphytic, saxicolous	18/47, 38%

* Presence of intron tested with PCR assays; All *T. decolorans* isolates were photobiont of epiphytic samples, while *T. arboricola* was identified from saxicolous specimens.

3.4.4 Introns of lichen photobionts and mycobionts

The origin of SSU 1512 group I introns in chlorophytes is still a matter of debate. Based on the observation of a close phylogenetic relationship of the 1512 group I intron lineage in chlorophytes to viral introns found in *Chlorella* spp., the viruses were hypothesized to be either the source or at least the vector, which facilitates the spread of group I introns among eukaryotes (Aimi et al. 1994; Bhattacharya, Friedl and Damberger 1996; Nishida et al. 1998).

Horizontal transfer of group I introns is known from many biological systems. Examples are 1) plant parasitic fungus to host plant (Nishida and Sugiyama 1995; Vaughn et al. 1995); 2) fungus to green alga (Lindstrom and Pistolic 2005); 3) fungus to red alga (Müller et al. 2005); 4) red alga to brown alga (Bhattacharya, Cannone, and Gutell 2001); 5) algae to amoebae (Turmel et al. 1995); 6) between eubacteria and chloroplasts (Kuhsel, Strickland, and Palmer 1990). However, no evidence has ever been obtained for intron transfer between the algal and fungal partners of lichens.

Only few insertion positions (516, 943, 1046, & 1506) for group I introns are found in both, green algae and ascomycetes (Gargas, Depriest and Taylor 1995). The SSU intron at position 1512, which is present on the surface of the mature ribosome in tertiary structure, is restricted to green algae (Gargas et al. 1995; Gargas, Depriest and Taylor 1995); it has never been found in fungi or other organisms. Depriest and Been (1992) concluded that group I introns of

lichen algae do not originate from their respective fungal partners or vice versa. Bhattacharya, Friedl and Helms (2002) demonstrated that there is no evolutionary relationship between group I introns of lichen-forming ascomycetes and their green algal partners; thus horizontal transfer can be excluded. Friedl et al. (2000) suggested that lichenization might facilitate the spread of 1512 introns among algal strains which coexist in fungal thalli. However, it is difficult to figure out how intron gain should proceed while the algal cells inhabit lichen thalli. In morphologically advanced, foliose or fruticose lichens the algal cells have physical contact only with sister cells derived from the same mother cell, but each of them is in direct contact with the fungal partner via the appressorial or haustorial complex (Honegger, 1991). A mycobiont-derived, water-repellent wall surface layer composed of hydrophobins and/or other hydrophobic wall surface components (Scherrer et al., 2000), ensheaths the algal and fungal surfaces in the thalline interior (Honegger, 1991; 2001). During the regular wetting and drying cycles solutes are passively exchanged underneath this hydrophobic sealing via the apoplastic continuum of both partners, but it seems unlikely that mobile genetic elements would be transmitted through this process. It is conceivable that intron gain occurs while lichen photobionts are not symbiotic, but free-living and in contact with cells of other species or strains. The example of the SSU 1512 group I intron of the type strain of *Trebouxia corticola*, as reported in this study, is consistent with this idea. This strain was isolated from free-living cells, not from a lichen, and its intron is similar to that in *Trebouxia decolorans* isolates from a different clade, the “arboricola” clade A (i.e., Helms 2003). According to the literature *Trebouxia arboricola*, the type species of the genus and photobiont of numerous lichen-forming ascomycetes, including Teloschistaceae, is also a common and widespread free-living alga (Ettl and Gärtner, 1995; John, Whitton and Brook, 2002; Rindi and Guiry 2003), despite contradictory claims (Ahmadjian 1988). The mechanisms underlying intron gain in lichen photobionts merit therefore a thorough investigation in future studies

3.5 Acknowledgements

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4 Genetic diversity among the sterile cultured *Trebouxia* photobionts from populations of *Xanthoria parietina* (lichen-forming ascomycete) visualized with RAPD-PCR fingerprinting techniques

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4.1 Abstract

Photobiont diversity within populations of *Xanthoria parietina* was studied at the species level by means of ITS analyses, and at the subspecific level with fingerprinting techniques (RAPD-PCR) applied to sterile cultured algal isolates. Populations from coastal, rural and urban sites from NW, SW and central France and from NE Switzerland were investigated. Between 8 and 63 samples per population, altogether 150 isolates, were subjected to different phenetic (NJ, UPJMA, SAHN) and ordination (PCA, PCOORDA) analyses. Epiphytic samples of *X. parietina* associated with different genotypes of *Trebouxia decolorans*, but saxicolous samples contained *T. arboricola*. For comparison the *T. gelatinosa* photobiont of a small population of *Teloschistes chrysophthalmus* (4 samples) was investigated. ITS sequences of *T. decolorans* isolates from different geographic locations were largely similar. In all populations a surprisingly high diversity of genotypes was observed among *Trebouxia* isolates from lichen thalli growing side by side. As *Trebouxia* spp. are assumed to be asexually reproducing haplonts the genetic background of this diversity is discussed. Fingerprinting techniques are a powerful tool for getting first insights in the genetic diversity within the algal partner of lichen-forming fungi at the subspecific level, provided that sterile cultured isolates are available.

4.1.1 Key words:

ITS RFLP, population genetics, *Teloschistes chrysophthalmus*, *Trebouxia arboricola*, *Trebouxia decolorans*, *Trebouxia gelatinosa*

4.2 Introduction

The photoautotrophic inhabitants of lichen thalli are distinctly less intensely studied than the lichen-forming fungi themselves. Before the advent of molecular techniques photobiont morphospecies were distinguished with light microscopy techniques. In case of the unicellular representatives of Trebouxiophyceae, most widespread photobionts of lichen-forming fungi, this was a difficult task. Prerequisite were sterile cultured isolates maintained under defined conditions and cultures of type species for comparison (Tschermak-Woess 1988). Later photobiont diversity within thalli of conspecific lichen-forming fungi was biochemically analysed, the focus being on isoenzyme patterns (Kilias 1988; Kilias *et al.* 1988; Fahselt 1989). With molecular techniques and algal-specific primers applied to whole lichen DNA the photobionts can be conveniently identified at species level without any isolation and culturing (Beck *et al.* 1998; Friedl *et al.* 2000; Dahlkild *et al.* 2001; Helms *et al.* 2001; Beck 2002). Thanks to this type of investigation our knowledge about the range of photobiont taxa associated with particular species of lichen-forming fungi has significantly increased in recent years. In most of these studies photobiont diversity was explored at species level on the basis or ITS phylogenies of samples collected in geographically different locations. In only few studies was photobiont diversity examined within populations. Photobiont diversity was studied in lichen communities growing on heavy-metal rich rock (Beck 1999; Beck *et al.* 1998; Beck *et al.* 2002). Photobiont diversity was explored in the families Cladoniaceae (Piercey-Normore & Depriest 2001) and Physciaceae (Dahlkild *et al.* 2001; Helms *et al.* 2001; Helms 2003), in the genera *Chaenotheca* (Tibell 2001), *Letharia* (Kroken & Taylor 2000), *Xanthoria* and *Xanthomendoza* (Nyati et al., unpubl.), and within populations of *Evernia mesomorpha* (Piercey-Normore 2006). In 290 thalli of this sorediate lichen-forming ascomycete sampled on 29 jack pine trees, five ITS genotypes of *Trebouxia jamesii*, revealing different restriction fragment length polymorphisms (RFLP), turned out to be the symbionts of two fungal ITS genotypes.

As ITS phylogenies are based on only one sequence area, genetic diversity at the subspecific level is best explored with fingerprinting techniques, i.e. with a multi-locus approach. Different fingerprinting techniques were successfully used for characterizing lichen-forming fungi at the subspecific level: microsatellite-based fingerprinting (Zoller *et al.* 1999; Walser *et al.* 2004; Walser *et al.* 2005; Walser *et al.* 2003), tRNA fingerprinting (Schmitt *et al.* 2002), or randomly amplified polymorphic DNA (RAPD) analysis applied to either pure fungal material such as central strands of *Usnea* sp. (Heibel *et al.* 1999) or apothecial discs (Printzen *et al.* 1999), or to sterile cultured fungal isolates (Murtagh *et al.* 1999; Dyer *et al.* 2001; Honegger *et al.* 2004b). RAPD-PCR applied to either randomly selected single spore isolates from one apothecium (Murtagh *et al.* 2000; Seymour *et al.* 2005) or to the single spore isolates derived from a single ascus (i.e. the progeny of one meiosis; Honegger *et al.* 2004a) was used for studying the mating systems of lichen-forming ascomycetes.

Fingerprinting techniques such as short (STRR) or long (LTRR) tandemly repeated repetitive sequences were used for characterizing conspecific cyanobacterial symbionts of angiosperms (Rasmussen & Svenning 1998), whilst M13 microsatellites facilitated the distinction of different genotypes among conspecific unicellular or filamentous green algae (Oppermann *et al.* 1997). The present study aims at elucidating photobiont diversity (*Trebouxia* spp.) at the subspecific level in different populations of the yellow wall lichen, *Xanthoria parietina*, by means of RAPD-PCR fingerprinting applied to sterile cultured isolates. Thalli were systematically sampled in populations at coastal, rural or urban sites in NW, SW or central France or in NE Switzerland. Some of these populations were old and undisturbed over prolonged periods of time (voucher numbers 120 and 121, 144 and 145), others grew at sites which had been newly built within the last 20 years (voucher numbers 111, 319 and 320). As *Trebouxia* species are assumed to reproduce exclusively asexually (Friedl & Büdel 1996) and be rare outside lichen thalli (Ahmadjian 1988) we were interested to see whether the photobionts of local populations of *Xanthoria parietina* are clonal or genetically diverse.

4.3 Materials and methods

4.3.1 Specimen collection, photobiont isolation and culturing

Lichen thalli from five populations of *Xanthoria parietina* and of one small population of *Teloschistes chrysophthalmus* were systematically collected (Table 4.1; Fig. 4.1A-B) and stored in desiccated state at -20°C where they stay viable for prolonged periods (Honegger 2003). Each collecting site and sample was photographically documented. Voucher specimens will be deposited in the herbarium of ETH Zurich (Z+ZT) after completion of ongoing projects. In the Swiss population (319/320) the thalli were left *in situ*, only small fragments being collected.

One, rarely two mature apothecia per thallus (in the latter case termed a and b) were selected and ascospores were allowed to be ejected for parallel experiments on the genetic diversity within the fungal partner (Honegger & Zippler, unpublished). In the herbarium sample the outline of each thallus was drawn on a transparent overlay and the site marked where apothecia had been removed. Photobiont cells were scraped out of the bottom (below subhymenial layer) and thalline margin of apothecia with a sterilized platinum needle and spread over the surface of agarized non-nutrient mineral medium (Bold's basal medium [BBM] according to Deason & Bold 1960) with double amount of nitrogen and 0.005% w/v Doxycycline (SIGMA) as an antibiotic. Plates were maintained at 15 ± 1°C at a 16h light/8 h dark cycle at approximate 5 µE m^{-2} s^{-1} for 2-3 weeks until cells started to divide. All cultures were screened regularly and fungal contaminants were removed. Dividing algal cells were transferred to *Trebouxia* medium II according to Ahmadjian 1967), with only ¼ amount of glucose and casmino acids (Honegger 2004). Most cultures are multi-cell isolates, cells originating from a very small area within a single apothecium, but few cultures are single cell isolates. A total of 150 photobiont isolates from five *X. parietina* populations were investigated in this study. For comparison 4 isolates of *Teloschistes chrysopthalmus* growing side by side were analyzed. In parallel experiments the fungal partner was brought in sterile culture.

Table 4.1 Collecting sites of lichen thalli and their *Trebouxia* photobionts isolated and analysed in the present study

Population‡ Collector	Collecting site Coordinates	Voucher No.	Substrate	Photobiont species*	ITS clade†	No. of isolates No. of markers
Xanthoria parietina						
SW France: Roussillon, Dept. Pyrénées-Orientales R. Honegger	highway stop Perpignan Sud; mediterranean**; urban** 42° 41' 55" N 02° 53' 44" E	111	*Celtis australis*	*Trebouxia decolorans*	n.d	7/37
SW France: Roussillon, Dept. Pyrénées-Orientales R. Honegger	Villefranche du Conflent**; rural** 42° 35' 0" N 02° 22' 0" E	120 121	*Populus* sp. *Prunus spinosus*	*T. decolorans* *T. decolorans*	Ac & others	25/44
Central France: BurgundyDept. Yonne R. Honegger	Relais fleuri near Avallon **; rural** 47° 29' 27" N 03° 54' 33" E	144 145	*Salix* sp. *Cornus albus*	*T. decolorans*	Ac	63/52
NW France: Brittany, Dept. Nord-Finistère R. Honegger	old port of Roscoff, Channel; **coastal** 48° 43' 08" N 03° 85' 48" W	164	*Myricaria germanica*	*T. decolorans*	Ac	8/25
NE Switzerland: city of Zürich S. Nyati & R. Honegger	University campus, Irchel; **urban** 47° 22' 0" N 08° 33' 0" E	319 320/1-/2 320/3	*Parthenocissus tricuspidatus* *Salix* sp. sandstone	*T. decolorans* *T. decolorans* *T. arboricola*	Ac Ac Ab	47/57
Teloschistes chrysophthalmus						
Spain: Canary Islands R. Gerber	La Gomera; **rural** 28° 06' 0" N 17° 08' 0" W	270	epiphytic	*T. gelatinosa*	Ib	4/26

‡Collecting sites from the same area are grouped as one population. * Photobiont species identified according to ITS and *rbc*L phylogenies; n.d. not determined. † ITS phylogenies in Fig. 4.3; “others” indicate *T. decolorans* isolates falling in unresolved part of phylogram between clade Ac and Ad.

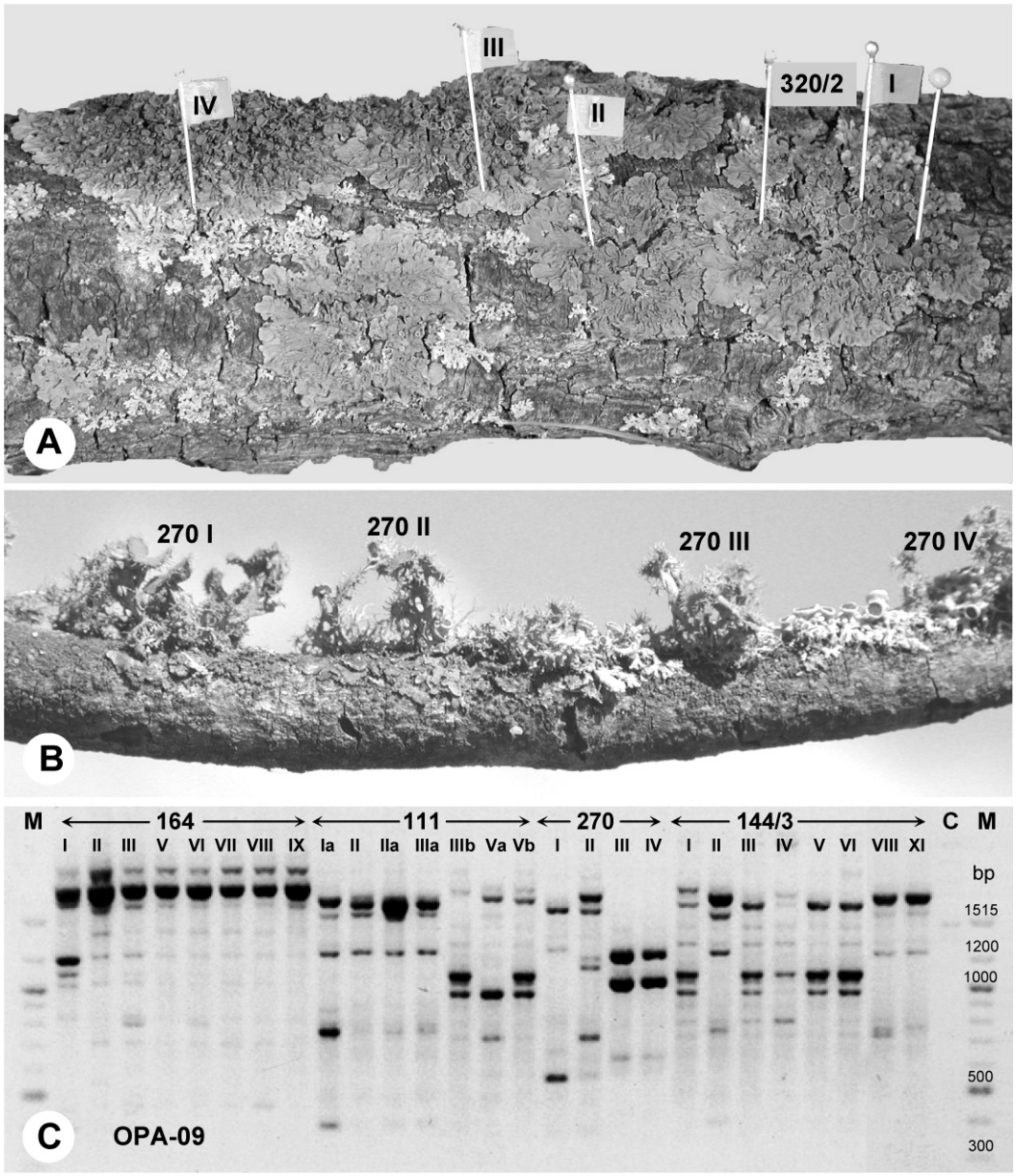

Figure 4.1 Population 320/2, with *Xanthoria parietina* growing on a vertical branch of *Salix* sp. at the pond in the University park Zürich Irchel. B, Population 270, *Teloschistes chrysophthalmus* growing intermixed with *Physcia* spp. on a vertical branch. C, RAPD-PCR of samples of populations 164, 111 and 144/3 (all *X. parietina*) and 270 (*Telo. chrysophthalmus*). C: control. M: molecular marker. See analysis of this sample set in Fig. 4D.

4.3.2 Genomic DNA isolation

Genomic DNA was isolated using the GFX PCR, DNA and Gel Band Purification Kit (Amersham Biosciences, Little Chalfont). Algal isolates were frozen in liquid nitrogen and ground with a pre-cooled motor-driven micropestle. After addition of 100 µl of capture buffer to ground material, the samples were incubated at 60°C for 10 mi-

nutes and subsequently centrifuged. The supernatant was transferred to a GFX column preloaded with 100 µl of capture buffer, incubated for 3 minutes at RT, centrifuged and washed with 500 µl of washing buffer. DNA was eluted in 50 µl of elution buffer (10 mM Tris-HCL, pH 8.0) and stored at 4°C.

4.3.3 ITS amplification, restriction digestion and sequencing

The nuclear ribosomal ITS region (ITS 1, 5.8S rDNA and ITS 2) was amplified with primer pair ITS 5 and ITS 4 (White *et al.* 1990). Internal primers at 5.8S rDNA were newly designed for sequencing (Nyati et al., unpubl.). For comparison three ITS sequences were obtained from whole lichen DNA extracts using forward primer AL1500bf (Helms *et al.* 2001) and reverse primer LR3 (Friedl & Rokitta 1997). Amplifications were performed in 50 µl reaction volume containing 2 µl genomic DNA, 50 nm each dNTP, 5 µl 10X PCR buffer, 0.6 µm each primers, 1.5U Taq DNA polymerase (Sigma) and 35.75µl autoclaved ddH_2O under the following conditions: initial denaturation at 95°C for 3', followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and elongation/extension at 72°C for 60 s and final extension at 72°C for 5'.

4.3.4 ITS sequencing and phylogenetic analysis

Sequencing was done in a 10 µl reaction mix containing 0.8 µl BigDye Terminator Mix V3.1, 120 nM primer, 1X reaction buffer, and 10-20 ng purified DNA. This reaction mixture was run as follows: initial denaturation at 94°C for 2 min, 60 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 3 min (0.9°C/s ramp). The products were analyzed on an Applied BioSystem/HITACHI ABI 3730 DNA Analyzer.

Sequences were analyzed with Sequencher^{TM} 4.2.2 (Gene Codes Corp. Ann Arbor, USA) and aligned automatically with Clustal X 1.81 (Thompson *et al.* 1997). The resulting alignment was manually aligned on MacClade 4.06 (Maddison & Maddison 2002). Phylogenetic analysis was carried out with PAUP 4.0 b10 (Swofford 1998) by Maximum Likelihood (ML), Maximum Parsimony (MP) and Neighbor Joining (NJ) methods. Bootstrap values for 1000 replicates were calculated separately. The sequence of *Trebouxia simplex* was used as outgroup.

4.3.5 ITS-RFLP

Only ITS-4 – ITS-5 amplified PCR fragments were used for ITS-RFLP. PCR products were either ethanol precipitated and concentrated prior to digestion, or small aliquots (10 µl) were directly subjected to restriction digestion with 4 units each of Eco RI, Hinc II, Hinf I and Pst I (New England Biolabs Inc., Boston, MA). Digestion reactions were incubated at 37°C for 2 hrs or overnight and heat inactivated at the temperature recommended by the manufacturer prior to loading on 1.2% agarose gel (Fig. 4.2A-C).

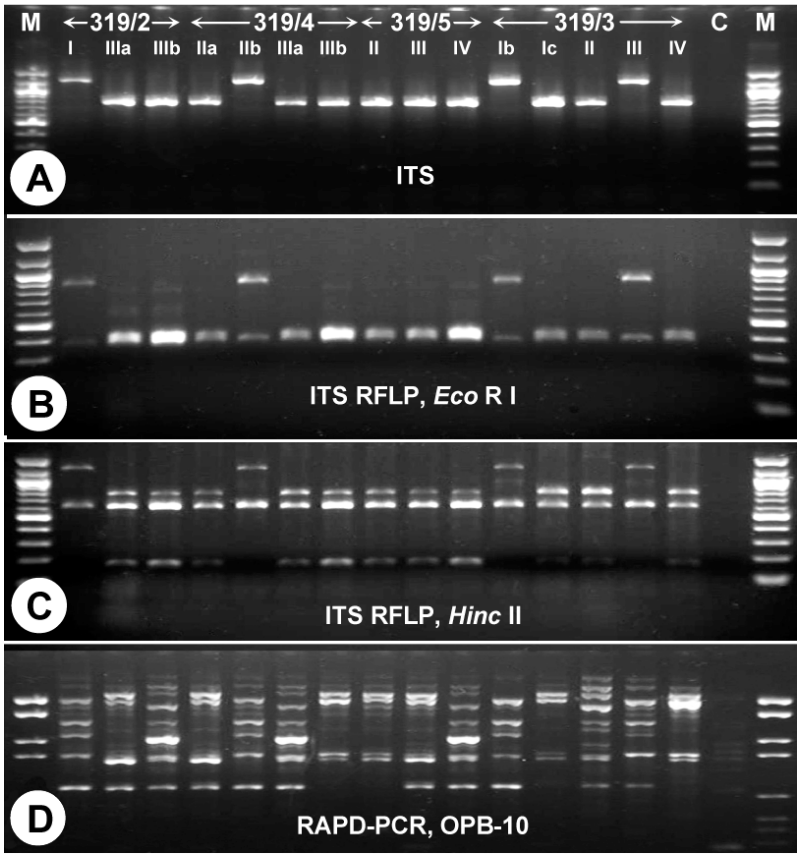

Figure 4.2 PCR products (ITS1 &2, 5.8S rDNA) from samples 319/2 – 319/5 on agarose gels. B) after digestion with *Eco* RI; C, after digestion with *Hinc* II. D, products of RAPD-PCR with primer OPA-09. C: control; M. molecular marker: 100 bp in A-C, Roche VI in D.

4.3.6 RAPD amplification and fingerprinting analysis

RAPD amplification was carried out with decamer primers (Operon Technologies Alameda, CA). Initial primer screening was carried out with 80 primers (Kit A-D) applied to DNA extracted from 2 *Trebouxia* isolates. 11 primers were found to be algal specific, but 5 of them gave only a very weak reaction. 38 primers amplified DNA derived from photobiont and mycobiont isolates. In the current investigation 4 primers (OPA-05: AGGGGTCTTG; OPA-09: GGGTAACGCC; OPB-10: CTGCTGGGAC, and OPC-06: GAACGGACTC) were used for amplification. Reactions were performed with the Perkin Elmer Gene Amp PCR system 9600. 25 μl reaction mix was prepared, containing 1 μl genomic DNA, 1X reaction buffer (Sigma, Buchs, Switzerland), 0.2 mM dNTPs, 1.25U Taq polymerase (Sigma), and 0.2 mM single primer. PCR conditions were as follows: initial denaturation for 3 min at 94°C, followed by 40 cycles of 30 s at 93°C, 40 s at 37°C and 80 s at 72°C with final extension of 5 min at 72°C. The ramp was 1°C/s. Negative controls were included in all experiments to detect contamination. 10 μl of each PCR product was loaded on 1.2% agarose gel and run for ~ 6 hrs for good separation of bands. DNA molecular weight marker VI (Roche Diagnostics GmbH, Mannheim) was used as a fragment size marker (Fig. 4.1C, 4.2D).

DNA from all algal isolates obtained from the same population was processed in the same run. Few selected isolates were independently amplified to check the reproducibility of band patterns. In three experiments the suitability of RAPD fingerprinting was checked using phenotypically similar single cell and multi-cell isolates from the same apothecia. Five algal isolates were sub-cloned into 5 isolates each and amplified to test uniformity of isolates. Only clearly visible strong bands were considered, their presence or absence being marked in a binary data matrix per primer and finally in a combined data matrix for each population or dataset. Isolates for which satisfactory amplification was not obtained with all primers were removed from the data matrix. This binary data matrix was then used for phenetic analysis (NJ and UPGMA) on FreeTree (Hampl *et al.* 2001; Pavlicek *et al.* 1999). The robustness of the resulting trees was tested with bootstrapping and jackknifing methods. Resulting phenograms were

analyzed on TreeView (Page 1996). Sequential agglomerative hierarchical nested cluster analysis (SAHN clustering) and ordination analysis were carried out on NTSYSpc (Rohlf 2000). In SAHN clustering, cophenetic-values were used to compute cophenetic correlation as a measure of goodness of fit, and results were plotted in the form of a dendrogram. In ordination analysis (principal components analysis, PCA and principal coordinates analysis, PCOORDA) both the variables and the points were plotted with respect to the same axes. In such biplots the patterns, trends etc. among the variables, but also the relationships between the points and variables are easily recognized.

4.4 Results

4.4.1 Suitability of methods

As shown in the ITS phylogram (Fig. 4.3) most of the *Trebouxia decolorans* isolates from samples collected in SW France (Roussillon), Burgundy and on our University campus in Zürich, Switzerland revealed identical or near identical ITS genotypes. Numerous isolates comprised a SSU 1512 group I intron of approx. 460 bases length (Fig. 4.2A), whose sequence was removed prior to alignment. In ITS RFLP experiments intron containing fragments gave different band patterns (Fig. 4.2B-C). A detailed analysis of the group I introns in *Trebouxia* photobionts of Teloschistaceae will be presented elsewhere (Nyati, Bhattacharya & Honegger, unpubl.). With the four enzymes used in this study only very few RFLP patterns could be resolved, but a total of 25 to 57 markers were obtained with four RAPD primers in our fingerprinting experiments (Tab. 4.1; Fig. 4.2D). Reproducibility was very high, provided that freshly isolated DNA was used and procedures standardized.

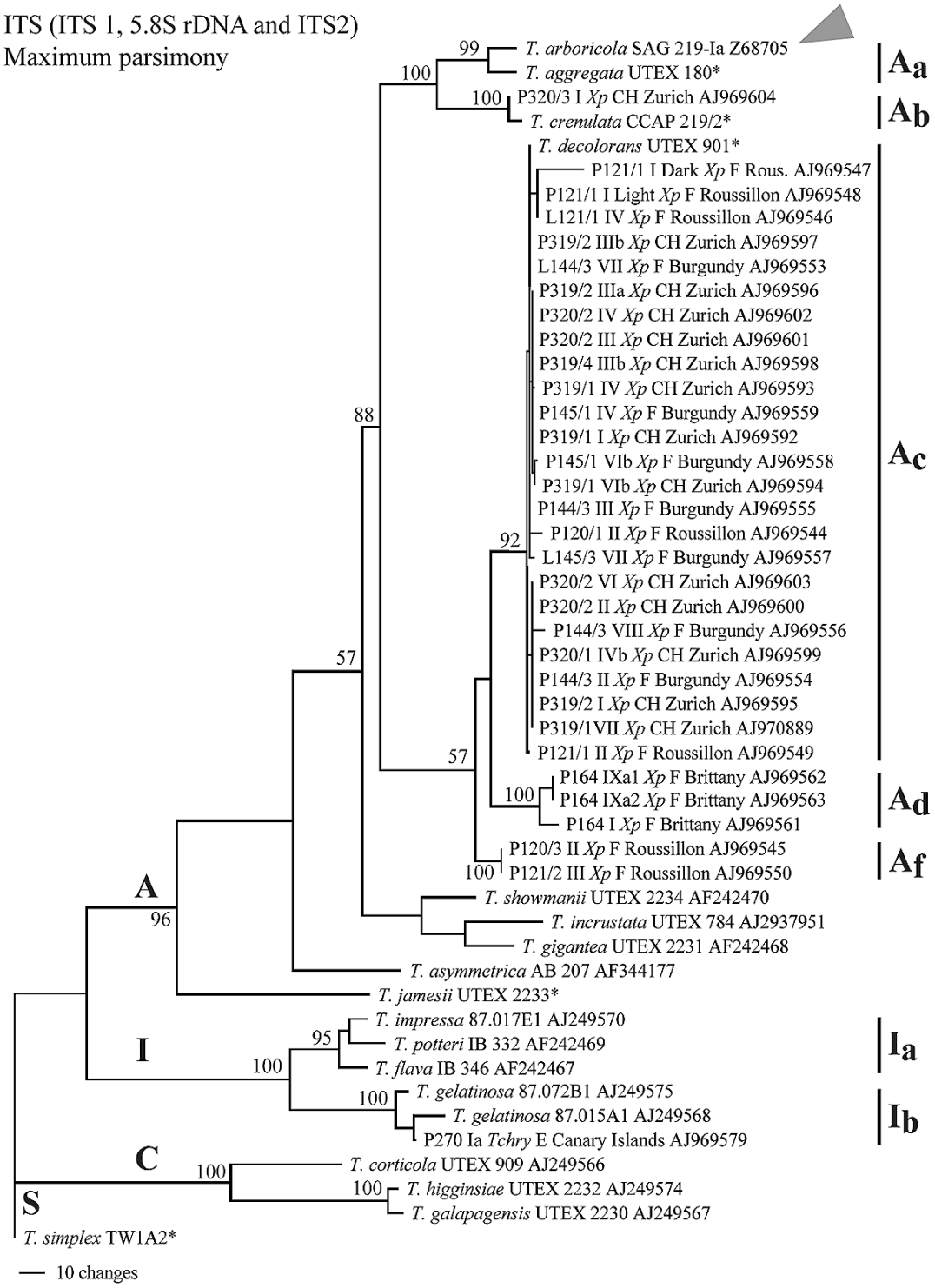

Figure 4.3 MP phylogram of internal transcribed spacer regions (ITS1 and ITS2) along with 5.8S rDNA. Bootstrap values for 1000 replicates are given at the nodes. The letters A, I, C, and S indicate 'arboricola', 'impressa', 'corticola' and 'simplex' clades respectively. The tree is outgroup rooted with *Trebouxia simplex* sequence. Type species of the genus (*T. arboricola*, SAG 219-Ia) is indicated with arrowhead. Unpublished sequences provided by T. Friedl and G. Helms are marked with asterisk. Each sample is labeled with voucher number followed by apothecia number, species abbreviation (*Xp*: *Xanthoria parietina*), country code, city/province and accession number. P denotes sequences obtained from a photobiont isolate while L indicates whole lichen DNA used for PCR amplification.

4.4.2 Population 120/121 from Roussillon (Dept. Pyrenées Orientales), SW France

4 thalli of *Xanthoria parietina* growing on 2 fragments (120/1, 120/3) of the same branch of *Salix* sp. and 11 thalli growing on 3 small branches of *Prunus spinosa* (121/1-121/3) were collected just outside the medieval city of Villefranche-du-Conflent, a famous tourist place, situated in a steep valley running in parallel to the Pyrenees in SW France near the Spanish border. These samples are termed “Roussillon” in ITS phylogram (Fig. 4.3), the local name of the larger area. All trees and shrubs at this site were completely covered by this golden-yellow lichen, indicating massive eutrophication. Both collecting sites were approx. 10 m apart from each other. All thalli revealed the same phenotype and contained *Trebouxia decolorans* as photobiont. From sample 121/1 I (one apothecium) four different algal phenotypes were isolated, which could be distinguished by their coloration: brown, green, light green and dark green. 2 different ITS genotypes were distinguished (Fig. 4.3). With fingerprinting techniques 3 different genotypes were identified among these 4 different algal phenotypes (Fig. 4.4A). This was the first and only time that different genotypes from the same algal species were isolated from a very small area, i.e. the thalline margin of an apothecium. In the remaining 14 samples 13 algal genotypes were found (Fig. 4.4A). Helms *et al.* 2001 found two different photobionts within single thalli of crustose Physciaceae (*Rinodina astrocinerea*, *R. tunicata* and *Rinodinella controversa*).

4.4.3 Population 144/145 from Burgundy, (Departement Yonne), Central France

22 Thalli of *Xanthoria parietina* were collected on 3 fragments of a branch of an old weeping willow (*Salix babylonica*) and 16 thalli on 3 twigs of a *Cornus albus* shrub in the garden of the mansion Le Relais fleuri near Avallon, situated within large fields adjacent to woods in Burgundy. All thalli of *X. parietina* revealed the same phenotype. ITS genotypes were very similar (Fig. 4.3). With fingerprinting techniques 36 different genotypes were identified among the 38 isolates of *Trebouxia decolorans* from this site (Fig. 4.4B).

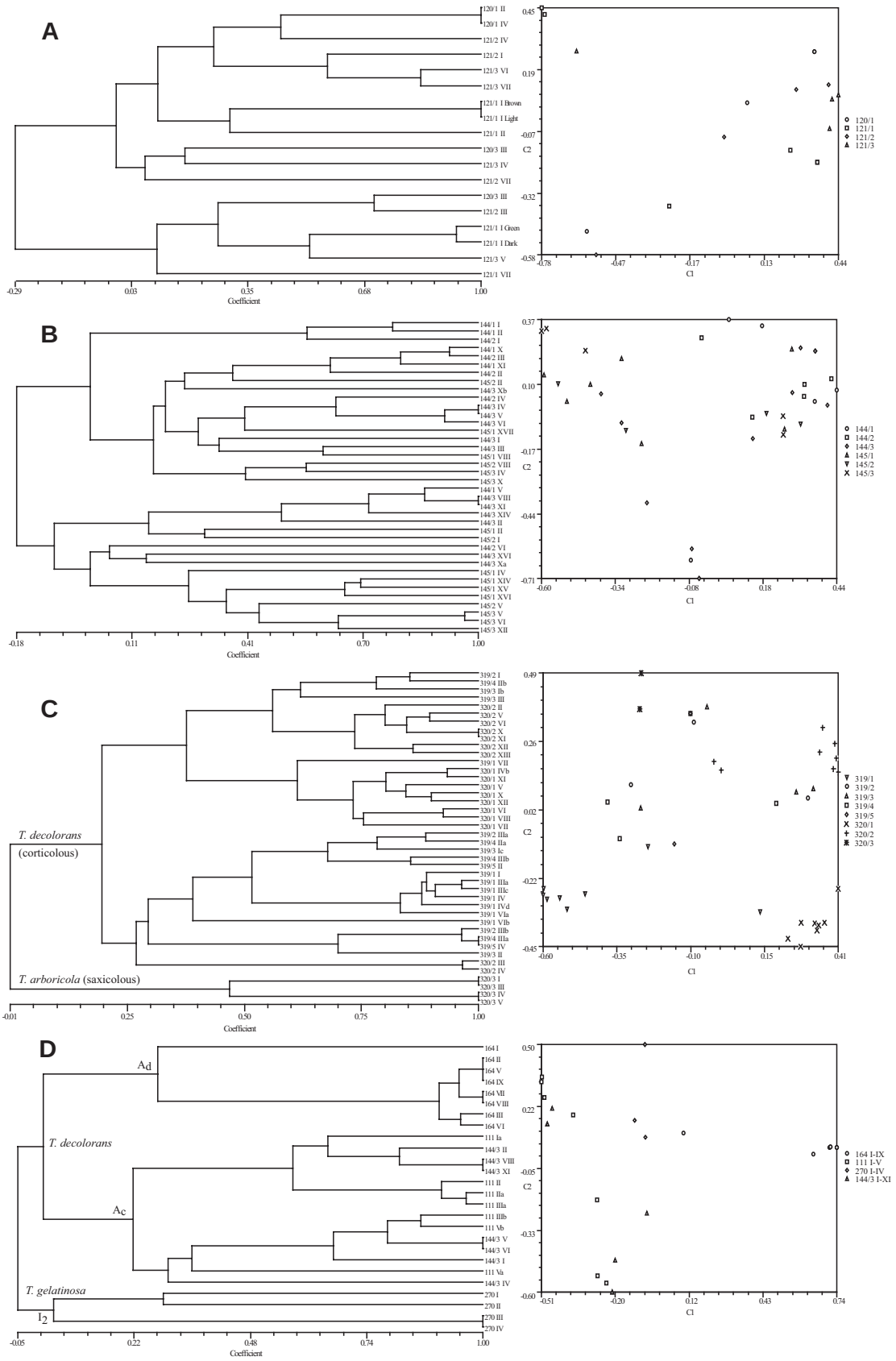
A
120/1 II
120/1 IV
121/2 IV
121/2 I
121/3 VI
121/3 VII
121/1 I Brown
121/1 I Light
121/1 II
120/3 III
121/3 IV
121/2 VII
120/3 III
121/2 III
121/1 I Green
121/1 I Dark
121/3 V
121/1 VII
-0.29
0.03
0.35
0.68
1.00
Coefficient
0.45
0.19
-0.07
-0.32
-0.58
C2
-0.78
-0.47
-0.17
0.13
0.44
C1
120/1
121/1
121/2
121/3
B
144/1 I
144/1 II
144/2 I
144/1 X
144/2 III
144/1 XI
144/2 II
145/2 II
144/3 Xb
144/2 IV
144/3 IV
144/3 V
144/3 VI
145/1 XVII
144/3 I
144/3 III
145/1 VIII
145/2 VIII
145/3 IV
145/3 X
144/1 V
144/3 VIII
144/3 XI
144/3 XIV
144/3 II
145/1 II
145/2 I
144/2 VI
144/3 XVI
144/3 Xa
145/1 IV
145/1 XIV
145/1 XV
145/1 XVI
145/2 V
145/3 V
145/3 VI
145/3 XII
-0.18
0.11
0.41
0.70
1.00
Coefficient
0.37
0.10
-0.17
-0.44
-0.71
C2
-0.60
-0.34
-0.08
0.18
0.44
C1
144/1
144/2
144/3
145/1
145/2
145/3
C
T. decolorans
(corticolous)
T. arboricola (saxicolous)
319/2 I
319/4 IIb
319/3 Ib
319/3 III
320/2 II
320/2 V
320/2 VI
320/2 X
320/2 XI
320/2 XII
320/2 XIII
319/1 VII
320/1 IVb
320/1 XI
320/1 V
320/1 X
320/1 XII
320/1 VI
320/1 VIII
320/1 VII
319/2 IIIa
319/4 IIa
319/3 Ic
319/4 IIIb
319/5 II
319/1 I
319/1 IIIa
319/1 IIIc
319/1 IV
319/1 IVd
319/1 Va
319/1 Vb
319/2 IIIb
319/4 IIIa
319/5 IV
319/3 II
320/2 III
320/2 IV
320/3 I
320/3 III
320/3 IV
320/3 V
-0.01
0.25
0.50
0.75
1.00
Coefficient
0.49
0.26
0.02
-0.22
-0.45
C2
-0.60
-0.35
-0.10
0.15
0.41
C1
319/1
319/2
319/3
319/4
319/5
320/1
320/2
320/3
D
Ad
Ac
T. decolorans
T. gelatinosa
I2
164 I
164 II
164 V
164 IX
164 VII
164 VIII
164 III
164 VI
111 Ia
144/3 II
144/3 VIII
144/3 XI
111 II
111 IIa
111 IIIa
111 IIIb
111 Vb
144/3 V
144/3 VI
144/3 I
111 Va
144/3 IV
270 I
270 II
270 III
270 IV
-0.05
0.22
0.48
0.74
1.00
Coefficient
0.50
0.22
-0.05
-0.33
-0.60
C2
-0.51
-0.20
0.12
0.43
0.74
C1
164 I-IX
111 I-V
270 I-IV
144/3 I-XI

Figure 4.4 Dendrograms obtained by SAHN clustering (left) and ordination of the first two axes of principal coordinate analysis (right). Each symbol represents an isolate. All except voucher numbers 270 are *Trebouxia* isolates from thalli of *Xanthoria parietina*. Details are given in the legend to the right. A, *Trebouxia decolorans* isolates from 'Roussillon' population (voucher numbers 120 on *Populus* sp., 121 on *Prunus spinosa*). B, *T. decolorans* isolates from Burgundy population (144 on *Salix babylonica*, 145 on *Cornus albus*). C, *T. decolorans* isolates from epiphytic populations (319 on *Parthenocissus tricuspidatus*, 320/1-320/2 on Salix sp.) and *T. arboricola* from saxicolous population (320/3) in Zürich. D, *T. decolorans* isolates from populations in Brittany (164 on *Myricaria germanica*), Perpignan (111 on *Celtis australis*), Burgundy (144/3 on *Populus* sp.) and *T. gelatinosa* from *Teloschistes chrysophthalmus* (270) from Canary Islands.

4.4.4 Population 164 from Brittany (Departement Nord-Finistère), NW France

8 thalli of *X. parietina* were collected on a tamarisk shrub at the easternmost mole next to the Chapelle Sainte Barbe in the old port of Roscoff at the Channel in NW France. This small population was unique because it comprised different thalline phenotypes with varying amounts of anthraquinone irrespective of illumination. Thalli growing side by side revealed colours from intense orange-yellow (164 I) to bright intense yellow (164 II) to greenish (164 V), some being greenish with intensely yellow lobe margin (164 IV). Juvenile thalli of *X. parietina* growing side by side on a fully illuminated wooden garden bench next to the *Myricaria* shrubs showed the same varieties of phenotypes. In TLC analyses all thalli of this population comprised parietin as main anthraquinone; thus belonging to chemosyndrome A according to Søchting (1997), characteristic feature of *X. parietina.* All thalli of *Xanthoria ectaneoides* growing on granitic rock at this collecting site revealed chemosyndrome A3 (Søchting 1997), with parietin, teloschistin and fallacinal as main anthraquinones (Honegger, unpubl.), and with *Trebouxia arboricola* as photobiont (Nyati et al., unpubl.).

Together with other *Trebouxia* isolates from *Xanthoria* spp. from maritime collecting sites on the Canary Islands, in the Eastern Mediterranean area and in Southern Australia the algal isolates of this population belong to a unique subclade Ad (Fig. 4.3) within clade A sensu Helms 2003; Nyati et al., unpubl.). With fingerprinting techniques 5 different genotypes were found among the 8 *Trebouxia* isolates from this site (Fig. 4.4), isolate Nr. 164/1 from the intensely orange coloured thallus, with different ITS genotype (Fig. 4.3), being distinctly different from all others (Figs. 4.1, 4.4.)

4.4.5 Population 111 from Perpignan (Departement Pyrenées Orientales), SW France

A small population comprising 7 thalli of *X. parietina* was collected on the stem of a *Celtis australis* tree at the stop of highway A9 (Narbonne to Barcelone) at Perpignan Sud in SW France. This site was newly constructed and trees planted less than 20 years ago. All thalli revealed an unusual phenotype with greenish gray colour and a slightly knobby surface, but in a phylogenetic analysis with multi-locus approach they all turned out to be normal *X. parietina* (C. Eichenberger, unpubl.). In mediterranean and also in continental climates the very smooth and hard bark of *Celtis australis* does not normally carry lichen growth, probably due to its very poor water holding capacity. Transplantation experiments would have to show whether these *X. parietina* thalli might differentiate a more typical phenotype on a different substrate. With fingerprinting techniques 7 genotypes were found among the 7 *Trebouxia* isolates from this site. None of them was sequenced, but as all isolates clustered with *T. decolorans* isolates from the Burgundy population 144, partly showing identical fingerprints (Fig. 4.4D), we conclude that they belong to the same species.

4.4.6 Population 319/320 from Zürich University campus, NW Switzerland

21 thalli of *X. parietina* were collected on the bottom part of 5 stems of a wild wine (*Parthenocissus tricuspidatus*; 319/1-319/5), 9 on the stem (320/1), 8 on a branch (320/2) of a willow tree (*Salix* sp.) at the pond and 4 on the sandstone underneath this willow tree (320/3) on our University campus at Zürich Irchel. All epiphytic samples comprised *Trebouxia decolorans*, all saxicolous samples *T. arboricola* as photobiont (Fig. 4.3, 4.4C). With fingerprinting techniques 36 genotypes were identified among the 38 epiphytic and 2 genotypes among the 4 saxicolous samples.

4.4.7 Population 270: *Teloschistes chrysophthalmus* from La Gomera (Canary Islands)

This small population of *Teloschistes chrysophthalmus* from La Gomera, Canary Islands, comprises only 4 thalli growing on the same branch (Fig.

4.1B). With fingerprinting techniques 3 distinctly different genotypes were found among the 4 isolates of *Trebouxia gelatinosa* (Fig. 4.1C). *T. chrysophthalmus* is usually richly fertile, but can also disperse vegetatively by means of the stiff marginal hairs at the tip of the fruticose thalli and at the apothecial margin, which break off very easily. They are predominantly fungal but comprise algal cells in their basal part.

4.5 Discussion

As shown in the present study RAPD-PCR is suitable for achieving first insights in the genetic diversity at the subspecific level in the green algal photobionts of lichen-forming fungi, provided that sterile-cultured isolates are available. As it was technically not possible to combine all data achieved in the present study in one large set, the different populations were analysed separately.

A combined analysis was carried out on 28 photobiont isolates from 3 different *X. parietina* populations (photobiont *T. decolorans*) and *T. chrysophthalmus* (photobiont *T. gelatinosa*) to test suitability of RAPD technique in differentiating algal genotypes originated from different *X. parietina* populations and differentiating *Trebouxia* species (Fig. 4.1) SAHN clustering dendrogram and PCOORDA analysis (Fig. 4.4) clearly grouped isolates into separate clades (clades Ac, Ad and Ib), as is the case in ITS phylogenetic analysis.

Each population of *Xanthoria parietina*, and even the small population of *Teloschistes chrysophthalmus* included in this study, turned out to comprise many different algal genotypes. In only few out of the 114 samples were identical fingerprints found. Some of them grew side by side (6 x 2 isolates), the others slightly apart from each other (3 x 2 and 1 x 3 isolates). The lowest diversity was found in the maritime epiphytic population 164 with 5 *Trebouxia* genotypes among 8 samples of *Xanthoria parietina*.

How can the surprisingly high genetic diversity among *Trebouxia* isolates within the different populations be explained? Geneticists primarily conclude on recombination (Kroken & Taylor 2000). However, sexual reproduction has neither been

demonstrated in the genus *Trebouxia* nor in other unicellular lichen photobionts of the genera of *Asterochloris*, *Coccomyxa*, *Dictyochloropsis*, *Myrmecia* etc. among the Trebouxiophyceae, which are all assumed to be asexual haplonts (Friedl & Büdel 1996). In the absence of sexual reproduction and recombination, genetic diversity results either from parasexual events, of which we have no knowledge in the algae concerned, or from mutations. Asexually reproducing organisms most likely accumulate more mutations over time than sexually reproducing ones. As long as their genome is stable and no house keeping genes are negatively affected by mutations, asexually reproducing species suffer no disadvantage over sexually reproducing ones. Examples of ancient asexual haplonts are the arbuscular mycorrhizal fungi (AMF), which have accumulated numerous mutations over time so that their multinucleate mycelia are heterokaryotic, i.e. harbor nuclei belonging to a wide range of different genotypes (Hijiri & Sanders 2004).

As *X. parietina* does not form symbiotic vegetative propagules, but lots of ascospores it is assumed to re-lichenized at each reproductive cycle. The present findings suggest that there is not a pool of one to few compatible algal genotypes present at each site, which might be accepted by ascospore-derived germ tubes, but many different genotypes. Alternatively, the populations of *X. parietina* and their *Trebouxia* photobiont, as investigated in the present study, might be not true populations as seen, e.g. in flowering plants, but an assembly of many different combinations of the fungal and algal partners, which were brought to the site from different external sources. *X. parietina* forms neither soredia nor isidia, but it does have options for vegetative dispersal in the symbiotic state. Thallus fragments are one possibility (Honegger 1996), lichenivorous invertebrates and their fecal pellets another one. Fecal pellets of the ever present oribatid mites, which are feeding on apothecia and thalli of *X. parietina*, grazing them down to the medullary layer, were shown to contain viable ascospores and *Trebouxia* cells (Meier *et al.* 2002). Migratory and other birds, passively carrying fecal pellets with them, were assumed to play a largely underestimated role in short and long distance dispersal of lichens (Meier *et al.* 2002).

An analysis of the genetic diversity among the fungal partner might show whe-

ther the *X. parietina* populations investigated in this study are mixtures of different genotypes from different sources. *Xanthoria parietina* was shown to be a self-fertile species, in which the progeny of meiosis is genetically identical with the mycelium of the mother thallus (Honegger *et al.* 2004a; Scherrer *et al.* 2005). In first RAPD-PCR experiments with ascospore-derived sterile cultured isolates of the respective fungal partners of our algal isolates Nr. 164 from coastal Brittany more fungal genotypes (7/8) were found (Honegger & Zippler, unpubl.) than among the algal isolates (5/8; this study). Fungal isolate 164 I, as algal isolate 164 I, turned out to be very different from all others. Thus combined data sets on the fungal exhabitant and the algal inhabitant of lichen thalli will bring interesting insights in population structure.

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5 Fine structure and phylogeny of green algal photobionts in the microfilamentous genus *Psoroglaena* (Verrucariaceae, lichen-forming ascomycetes)

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5.1 Abstract

According to the literature the microfilamentous thalli of lichen-forming ascomycete genus *Psoroglaena* are assumed to harbour vivid green “prochlorophyte” cyanobacterial photobionts. As this would be the first report of terrestrial “prochlorophytes” we investigated the fine structure and two molecular markers (SSU rDNA and rbcL) of the photobionts of *P. stigonemoides* (Orange) Henssen and *P. epiphylla* Lücking. Both *Psoroglaena* spp. had unicellular green algal photobionts, representatives of the Trebouxiophyceae. The photobiont of *P. stigonemoides* is closely related to the non-symbiotic *Auxenochlorella protothecoides* and to a *Chlorella* endosymbiont of the freshwater polyp *Hydra viridis*. The photobiont of *P. epiphylla* is related to *Chlorella luteoviridis, C. saccharophila* and a *Pseudochlorella* isolate. In contrast to other microfilamentous lichens, which derive their shape from filamentous green algae or cyanobacterial colonies overgrown and ensheathed by the fungal partner, *Psoroglaena* mycobionts position their unicellular photobiont in uni-or multiseriate rows which strongly resemble the situation in filamentous cyanobacterial colonies.

5.1.1 Key words

Auxenochlorella protothecoides, *Chlorella luteoviridis*, *Psoroglaena epiphylla*, *Psoroglaena stigonemoides*, prochlorophytes, SSU rDNA, rbcL.

5.2 Introduction

Lichen-forming asco- and basidiomycetes differentiate a wide array of symbiotic phenotypes, ranging from internally non-structured crustose thalli on or within the substratum to highly advanced, internally stratified and morphologically complex foliose or fruticose thalli. In morphologically advanced lichens the fungal partner rises above the substratum, competes for space and actively positions the photobiont cell population in the thalline tissue where optimal conditions are found for photosynthesis (Honegger, 1991). Unicellular or microcolonial photobionts of morphologically advanced lichen-forming fungi trigger, in a yet unknown manner, the phenotypic expression of the fungal genotype (Honegger, 1993), but they have no direct impact on thallus shape and structure. A different situation occurs in microfilamentous lichens, as formed by various asco- or basidiomycetes with either green algal or cyanobacterial photobionts. These thalli are formed by fungal hyphae which ensheath or overgrow a filamentous photobiont, the latter presetting or at least strongly influencing the final shape of the thallus. Microfilamentous thalli are formed by lichen-forming ascomycetes in association with filamentous cyanobacteria of the genera *Stigonema* (lichen genera *Ephebe*, *Spilonema*, *Zahlbrucknerella* etc.), *Calothrix* and *Dichothrix* (genus *Lichina*) or *Scytonema* (genera *Lichenothrix*, *Thermutis* etc.), or by lichen-forming basidiomycetes in association with *Scytonema* sp. (genus *Dictyonema*). Filamentous green algae, representatives of the *Trentepohliaceae*, form the “backbone” of microfilamentous thalli of the ascomycetous genera *Cystocoleus*, *Coenogonium* and *Racodium* (Koch, 1961; Henssen, 1963; Henssen and Jahns, 1973; Tschermak-Woess, 1988; Friedl and Büdel, 1996).

Very peculiar microfilamentous phenotypes are formed by lichen-forming ascomycetes of the genus *Psoroglaena* (Müller Argoviensis, 1891), whose minute, corticolous or foliicolous, vivid green thalli (Figs. 5.1a, f) are easily confused with free-living filamentous green algae or with moss protonemata, all of which may form extensive velvety colonies on their respective substrata. *Psoroglaena* spp. invariably grow in mild, very humid habitats in temperate or subtropical to tropical areas, i.e. at sites where free-living filamentous green algae and moss protonemata are common. For more than hundred years after its description *Psoroglaena*

cubensis Müll.Arg. was the only species known from this genus, which was collected only once in Cuba. It was refound in Cuba, then discovered in samples from Paraguay, USA, Australia, the Seychelles and Kenya by Eriksson (1992). In the meantime a morphologically similar European species was described under the name *Macentina stigonemoides* (Orange, 1989). Based on characteristics of the ascomata it was included in the genus *Macentina* (Vedza, 1973), which contains mainly crustose species. The genus *Macentina* originally comprised species that were mostly living on leaves and form minute ascomata, *Leucocarpia abscondita* (Coppins & Vìzda) Hafellner and *Leucocarpia stigonemoides* (Orange) Hafellner & Kalb, the latter being synonymous with *Macentina stigonemoides* Orange, were transferred to the genus *Psoroglaena* (Hafellner & Kalb 1992; Hafellner & Maurer 1994; Henssen 1995). All three genera (*Leucocarpia*, *Macentina* and *Psoroglaena*), which are now treated under *Psoroglaena*, share light coloured ascomata, but there are doubts that they all represent a single monophyletic group. All three genera belong to the Verrucariaceae. Today 12 *Psoroglaena* species are described, most of them living in tropical rainforests, mainly foliicolous on long-living leaves (Hafellner, 2002; Aragon and Sarrion, 2003; Harada, 2003; Herrera-Campos et al., 2004a, b).

Divergent views are found in the literature concerning the taxonomic affiliation of the photobionts of *Psoroglaena* spp., which give the thalli their vivid green colouration. Müller Argoviensis (1891), Orange (1989) and Eriksson (1992) described them as unicellular green algae. Henssen (1995) concluded on the basis of light microscopic investigations that the photobionts of *Psoroglaena* spp. are filamentous blue-green algae (cyanobacteria) with no heterocysts, but with a yellow-green pigmentation. She assumed them to be undescribed representatives of the “prochlorophytes” similar to the microfilamentous freshwater planctontic *Prochlorothrix hollandica*. If true, this would be the first representative of “prochlorophyte” cyanobacteria ever detected in terrestrial ecosystems, a quite spectacular finding. We therefore were interested to investigate the photobionts of *Psoroglaena* spp. with ultrastructural and molecular techniques. “Prochlorophytes” reveal all features of cyanobacteria except their pigmentation: cyanobacteria have chlorophyll a and phycobilins, whereas “prochlorophytes” contain chlorophyll a and b, but lack phycobilins. Thus they are vivid green

instead of greyish to turquoise or pinkish, as normally seen in cyanobacteria. “Prochlorophytes” were speculated to be modern counterparts of the ancestors of the green chloroplast of plants (Lewin, 1976), but phylogenetic analyses did not support this hypothesis (Turner et al., 1989; Shimada et al., 1995; Palenik and Swift, 1996).

5.3 Materials and Methods

5.3.1 Collecting sites and isolation of the photobiont

Psoroglaena stigonemoides was collected in Tintern, Wales (UK) by A. Orange, October 1995. *P. epiphylla* was collected on the volcano San Martín Tuxtla, Veracruz (Mexico) by R. Lücking, February 2003. Both samples were sent to R. H. soon after collecting and drying. Part of the specimens was immediately processed for electron microscopic investigations, the rest was kept, in the desiccated state, at -20°C in our laboratory. The voucher specimens will be deposited in part in the herbarium of ETH Zürich (ZT) and Botanische Staatssammlung München, Munich (M).

The photobiont of *P. stigonemoides* was successfully isolated twice into sterile culture. Once by R. H. from a single tip cell of a thalline microfilament and a second time by A. B. from a cell within the thallus using a single-cell manipulator as described in Beck and Koop (2001). The isolates are kept on *Trebouxia* medium according to Ahmadjian, 1967), but with only ¼ of the C and N sources (Honegger, 2004). Both isolates were found to be morphologically identical yielding identical ITS nrDNA sequences and are thus not distinguished below. All attempts to isolate the photobiont of *P. epiphylla* failed, and none of the fungal partners could be isolated into sterile culture. Thus whole lichen DNA extracts were used for PCR amplification of the photobiont of *P. epiphylla*.

5.3.2 Light and electron microscopy

Light microscopy. Habitus images were taken with a NIKON Coolpix 990 camera connected to a WILD M5A dissecting microscope. Bright field light micrographs of specimens mounted in water were taken with the same camera connected to

a ZEISS Photomikroskop II.

Scanning electron microscopy (SEM). Dry specimens were rehydrated in a humid chamber for at least 2 h prior to chemical fixation in the vapour of a 4% OsO_4 solution. Specimens were dehydrated in an ascending series of acetone, starting with 50% in H_2O, then critical point dried, mounted, sputter-coated with gold and examined in a HITACHI Stereoscan field emission microscope.

Transmission electron microscopy (TEM). Dry specimens were rehydrated overnight. Chemical fixation was performed in 1.25% glutaraldehyde and 1% acrolein in phosphate buffer (pH 7.1) for 4 h at RT, postfixation in 2% OsO_4 in buffer overnight. After dehydration in an ascending series of acetone the specimens were slowly infiltrated with Spurr's low viscosity epoxy resin. Ultrathin sections cut with a DIATOME knife on a REICHERT OM U3 ultramicrotome were stained with uranyl acetate and Sato's lead mixture, then examined in HITACHI H 7000 transmission electron microscope.

5.3.3 DNA isolation, PCR amplification and sequencing

DNA was isolated either from the sterile cultured photobiont or whole lichens using GFX PCR, DNA and Gel Band Purification Kit (Amersham Biosciences, Little Chalfont) following the protocol of manufacturer. Algal isolate or lichen material was frozen in liquid nitrogen and ground with a pre-cooled motor-driven micropestle. After addition of 100 µl of capture buffer to ground material, the samples were incubated at 60°C for 10 minutes and subsequently centrifuged. The supernatant was transferred to GFX column preloaded with 100 µl of capture buffer, incubated for 3 minutes at RT, centrifuged and washed with 500 µl of washing buffer. The DNA was eluted in 50 µl of elution buffer (10 mM Tris-HCL, pH 8.0) and stored at 4°C.

P. epiphylla photobiont SSU was amplified with newly designed primer pair SSU fwd (GACTCGCGGTGACTCATGATAAC) and SSU rev (YTTCACCAGCACAYYCAATCG), 3' ends located at positions 247 and 1685 of the reference sequence X56101 (*Auxenochlorella protothecoides*), respectively. An additio-

nal primer pair was designed for sequencing (SSU fwd1 GCAAGTCTGGTGCCAGCA and SSU rev1 AATCGGTAGGAGCGACGGGCGG, 3' ends located at positions 553 and 1669 of the reference sequence X56101, respectively). SSU of the *P. stigonemoides* photobiont isolate was amplified using forward primer 82F (Schmitt and Lumbsch, 2001), and reverse primer B (Medlin et al., 1988), which was used without the polylinker site. PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 36 cycles of 95°C for 40 s, 56°C for 40 s, and 72°C for 1.2 min with final extension at 72°C for 10 minutes. PCR products were either sequenced directly on a Licor 4200 automated sequencer as described by Beck, 1999 or cloned before sequencing. Cloning was performed using pGEM®-T Easy Vector System (Promega Corp., WI, USA) and competent XL10-Gold® *Escherichia coli* cells (Stratagene, CA, USA). Plasmid DNA was isolated using GFX™ Micro Plasmid Prep Kit (Amersham Biosciences, NJ, USA).

The chloroplast-encoded large subunit (*rbc*L) of the Rubisco gene was amplified with the primers as described in Nyati et al. (submitted). PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 30 cycles (95°C for 45 s, 52°C for 60 s, and 72°C for 80 s), with final extension at 72°C for 10 min. The PCR product of *P. epiphylla* was cloned prior to sequencing, while the PCR product of the *P. stigonemoides* photobiont isolate was sequenced directly after gel elution.

Sequencing was performed in 10 µl reaction mix containing 0.8 µl BigDye Terminator Mix V3.1, 1X reaction Buffer, 120 nM primer and purified PCR fragments (10-20 ng DNA) or plasmid (150-300 ng DNA) with the following setting: initial denaturation at 94°C for 2 min, followed by 60 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 3 min (0.9°C/s ramp). These products were analyzed on an Applied BioSystem/HITACHI ABI 3730 DNA Analyzer.

5.3.4 Phylogenetic analysis

Sequence contigs were aligned and analysed with Sequencher™ 4.5 (Gene Codes Corp. Ann Arbor, USA.). Unambiguous sequences were aligned automatically on ClustalX (Thompson et al., 1997) with the algal isolates used for comparison

(listed in Table 4.1), and the resulting matrix of aligned sequences was manually refined in MacClade 4.06 (Maddison and Maddison, 2002). Ambiguous regions of the alignment were excluded from the analysis. Maximum parsimony (MP), Maximum Likelihood (ML) and Neighbour joining (NJ) analysis were carried out using Paup 4.0b10 (Swofford, 1998). Weighted Parsimony and NJ analysis were performed as described by Friedl, 1995. For the ML analysis the model fitting the data best has been determined using Modeltest 3.7 (Posada and Crandall, 1998) and the optimal model selected under the Akaike information criterion was used (GTR+I+G; Tavarı, 1986). Jackknife values for 500 (MP and NJ analyses) or 100 (ML analyses) replicates were calculated separately.

Table 5.1 Algal strains and lichen photobionts used in present study along with SSU and rbcL accession numbers

Algae/photobiont	strain/isolate	in symbiosis with‡	SSU accession #	*rbc*L accession #	reference*
Asterochloris erici	IB342			AJ969631	Nyati et al., submitted
Asterochloris magna	UTEX 902	*Pilophorus acicularis*	Z21552.1		Friedl and Zeltner, 1994
Chlamydomonas reinhardtii			M32703.1		Gunderson et al., 1987
Chlamydomonas sp.	ICE-W			AY731087	Liu et al., 2006
Chlorella ellipsoidea	SAG 211-1a		X63520.1		Krienitz et al., 1996
Chlorella ellipsoidea	C-27			D10997	Yoshinaga et al., 1988
Chlorella lobophora	Andreyeva 750-I	*Hydra viridissima*	X63504.1		Huss et al., 1993/94
Chlorella luteoviridis	SAG 211-2a		X73997.1		Huss et al., 1999
Chlorella minutissima	SAG 1.80		AB006046		Hanagata N (unpubl.)
Chlorella mirabilis	Andreyeva 748-I		X74000.1		Krienitz et al., 1996
Chlorella protothecioides var. *acidicola*	124		AJ439399		Huss et al., 2002
Auxenochlorella protothecoides	SAG 211-7a		X56101.1	AM260440	Huss and Sogin, 1990; this study
Chlorella saccharophila	SAG 211-9a		X63505.1		Krienitz et al., 1996
Chlorella saccharophila	SAG 211-1a			AM260446	This study
Chlorella sorokiniana	Prag A14		X74001.1		Huss et al., 1999
Chlorella sp. ex *Hydra4*		*Hydra viridissima* Ssh	X72854		Huss et al., 1993/94

Chlorella sp. ex *Hydra6*		*Hydra viridissima* Esh	X72706		Huss et al., 1993/94
Chlorella sp. ex *Paramecium6*		*Paramecium bursaria* Cs2	AB206546		Hoshina et al., 2005
Chlorella sp. ex *Paramecium8*	CCAP 1660/11	*P. bursaria*	AB206548		Hoshina et al., 2005
Chlorella sp.	SAG 211-18		X73992.1		Huss et al., 1999
Chlorella sp. *ex Hydra8*		*Hydra viridissima* Jsh	X72708.1		Huss et al., 1993/94
Chlorella vulgaris	SAG 211-11b		X13688	AF499684	Huss and Sogin, 1990; Hayden and Waaland, 2002
Chlorococcum hypnosporum	UTEX 119		U41173.1		Nakayama T et al (unpubl.)
Choricystis minor	SAG 251-1		X89012.1		Krienitz et al., 1996
Choricystis sp.	WTwin9/21P-7W			AY902235	Fawley and Fawley (unpubl.)
Coccobotrys verrucariae	SAG 16.97			AM260447	This study
Dictyochloropsis reticulata	CCHU 5616		Z47207.1		Friedl, 1995
Diplosphaera mucosa	SAG 48.86			AM260444	This study
Diplosphaera sp.	SAG 49.86			AM260445	This study
Dunaliella salina	UTEX 1983		M84320.1		Wilcox et al., 1992
Elliptochloris bilobata	SAG 245.80				This study
Enteromorpha intestinalis	SY0104		AJ005413.1		Tan & Sluiman (unpubl.)
Haematococcus pluvialis	SAG 34-1b		AF159369.1		Hepperle et al., 1998
Leptosira obovata	SAG 445-1		Z68695.1		Friedl, 1996
Leptosira terrestris	SAG 463.2			AM260448	This study

Microthamnion kuetzingianum	UTEX 1914		Z28974.1		Friedl and Zeltner, 1994
Muriella terrestris	ASIB V38		AB012845.1		Hanegata, 1998
Myrmecia biatorellae	UTEX 907	*Dermatocarpon tuckermani*	Z28971.1		Friedl and Zeltner, 1994
Myrmecia biatorellae	SAG 8.82			AF499685	Hayden and Waaland, 2002
Nanochlorum eucaryotum	Mainz 1		X06425.1		Sargent et al., 1988
Parachlorella kessleri	IAM C-531		AB080309		Yamamoto et al., 2003
PB Psoroglaena epiphylla	L-1016	*P. epiphylla*	AM260450		This study
Psoroglaena epiphylla epibiont				AM260443†	This study
PB Psoroglaena stigonemoides	P-1015	*P. stigonemoides*	AM260449	AM260439	This study
Prasiola calophylla	Galway			AY694194	Rindi et al., 2004
Prasiola crispa	SAG 43.96		AJ416106	AM260441	Friedl and O'Kelly, 2002; this study
Prasiola fluviatilis			AF189072		Sherwood et al., 2000
Prasiola furfuracia				AF189064	Sherwood et al., 2000
Prasiola mexicana	MEX12		AF189075		Sherwood et al., 2000
Prasiolopsis ramosa	SAG 26.83		AY762600		Karsten et al., 2005
Prototheca wickerhamii	SAG 263-11		X74003.1		Huss et al., 1999
Prototheca zopfii	SAG 263-1a		X63519.1		Huss et al., 1999
Pseudochlorella sp.	CCAP 264-2		AB006049		Hanagata N (unpubl.)
Raphidonema longiseta			U18520		Chapman R L (unpubl.)
Rosenvingiella polyrhiza	GC			AY694206	Rindi et al., 2004

Stichococcus bacillaris	UTEX 314		U18524.1		Rootes & Chapman (unpubl.)
Stichococcus bacillaris	K4-4		AB055866		Hanagata N (unpubl.)
Stichococcus bacillaris	SAG 379-1b			AM260442	This study
Stichococcus chlorelloides	BCP-CNP2VF11B		AY271675.1		Lewis and Lewis, 2005
Trebouxia arboricola	SAG 219-1a	Type species, free-living!	Z68705.1	AM158960	Bhattacharya et al., 1996; Nyati et al. (submitted)
Ulva rigida	EL0102		AJ005414.1		Tan and Sluiman (unpubl.)
Volvox carteri	UTEX 1885		X53904.1		Rausch et al., 1989
Watanabea sp.	SAG 211-9b		X73991.1		Huss et al., 1999

* When two references are provided, the first reference refers to the SSU sequence, the second to the *rbc*L sequence.
‡ when organism name not given, the alga found free-living.
† This *rbc*L sequence most likely belongs to an epibiont growing on *Psoroglaena epiphylla.*

5.4 Results

5.4.1 Ultrastructural data

As clearly seen in TEM micrographs (Figs. 5.1d-e, g-h) the photobionts of *Psoroglaena stigonemoides* and *P. epiphylla* are not cyanobacteria (prokaryotes), but unicellular eukaryotes with one parietal chloroplast per cell and a weakly visible pyrenoid in the centre of the chloroplast. In the symbiotic state the photobiont of *P. epiphylla* had a very large, cup-shaped parietal chloroplast which makes up a high proportion of the total volume of the cell. The nucleus is located in the centre of the cell (Fig. 5.1e). In their youngest, tip-most parts the microfilamentous thalli of *P. epiphylla* were slim, the photobiont cells being arranged in uniseriate rows and tightly ensheathed by the mycobiont (Figs. 5.1b-d). Knobby outgrowths of the peripheral hyphal wall (Figs. 5.1b, d-e) are a characteristic feature of most *Psoroglaena* species. In *P. stigonemoides* the photobiont cells were arranged mostly in multiseriate rows, the fungal partner growing around and between the algal cells. Only at the very tip of the microfilamentous thallus were algal cells often arranged in single rows. The parietal chloroplast contained numerous plastoglobules in its central part (Fig. 5.1g-h), marking the location of the weakly visible pyrenoid.

In TEM peparations the specimen preservation in both *Psoroglaena* spp. turned out to be less than optimal, both mycobiont and photobiont being affected. This preparative procedure (Honegger and Brunner, 1981) was successfully used for numerous studies on lichens and other fungal symbioses with photoautotrophic partners. *Psoroglaena* spp. and their photobionts might be among the few lichens so far known to science which do not tolerate desiccation for prolonged periods of time. This could also be the reason why isolation and culturing experiments largely failed, with the exception of two photobiont cells of *P. stigonemoides*. Lack of drought tolerance in the fungal and photoautotrophic partners might be common among lichens of constantly humid ecosystems such as tropical rainforests.

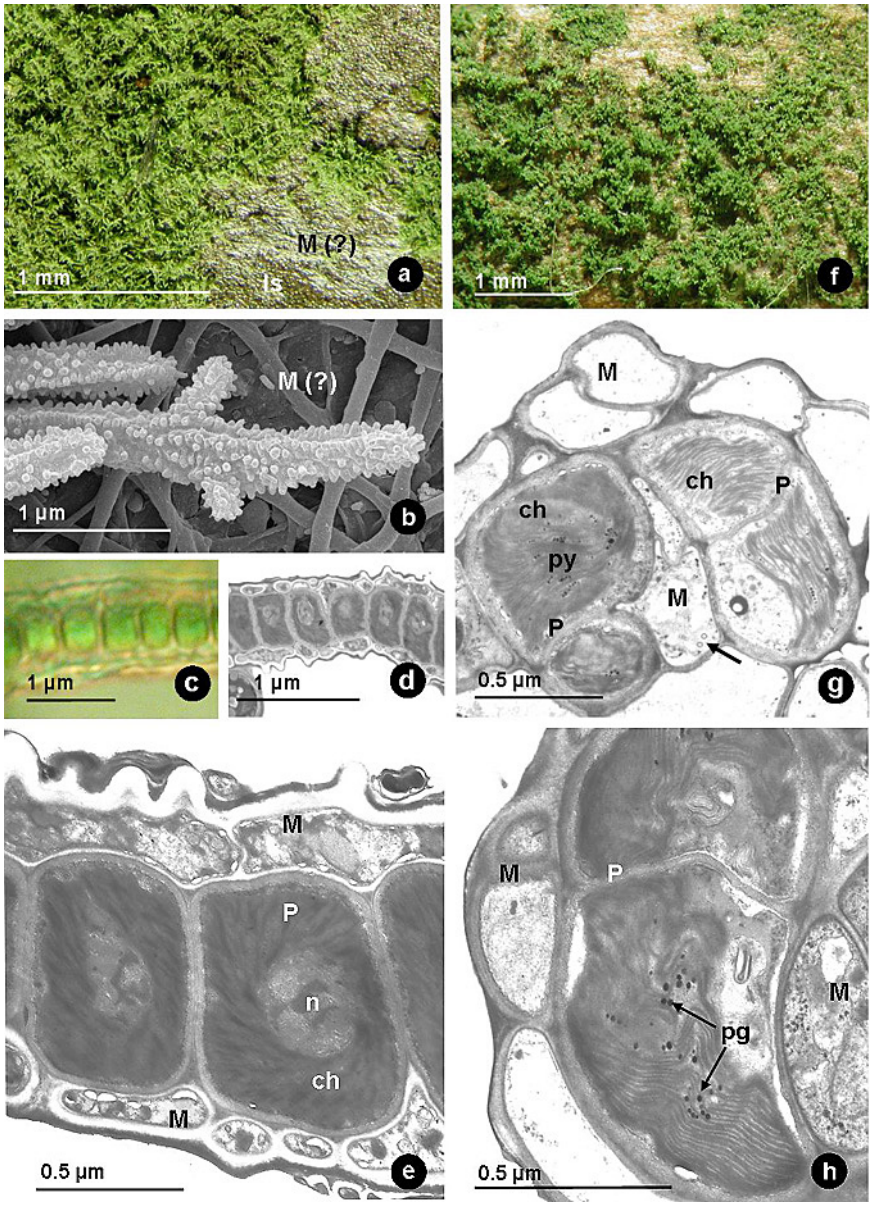

Figure 5.1 Light and electron micrographs of *Psoroglaena epiphylla* (a-e) and *P. stigonemoides* (f-h). a), f) *Psoroglaena* spp. as seen with a dissecting microscope. The vivid green, microfilamentous thalli resemble either filamentous green algae or moss protonemata. a) *P. epiphylla* growing on a leaf surface (ls). Whitish mycelium at the leaf surface is assumed to be the aposymbiotic mycobiont. b) Scanning electron micrograph of part of the microfilamentous thalli at the periphery of the colony, with knobby outgrowths of the wall surfaces of lichenized hyphae. Non-lichenized hyphae with smooth wall surfaces, presumably of the mycobiont, grow along the leaf surface. c) Light micrograph of an uniseriate microfilamentous thallus, with vivid green photobiont cells ensheathed by parallel fungal hyphae. d) Transmission electron (TEM) micrograph of an uniseriate microfilamentous thallus. e) detail of d). The green algal photobiont cells have one parietal chloroplast (ch). The nucleus (n) is located in the centre of the cell. f) *P. stigonemoides* growing on dead wood. g) TEM micrograph of part of a multiseriate microfilamentous thallus. The parietal chlorplast (one per cell) contains a pyrenoid (py). The arrow points to two concentric bodies in the fungal cytoplasm. h) detail of a photobiont cell ensheathed by the mycobiont. Arrows point to groups of plastoglobules (pg) in the chloroplast.

Abbreviations: ch, chloroplast; ls, leaf surface; M, mycobiont; M(?) presumably aposymbiotic mycobiont; n, nucleus; P, photobiont; pg, plastoglobules; py, pyrenoid.

5.4.2 SSU phylogeny

Molecular data sets clearly show that the photobionts of *Psoroglaena epiphylla* and *P. stigonemoides* are green algae belonging to the class Trebouxiophyceae, which harbours a wide range of lichen photobionts. The consensus trees obtained by the MP, ML and NJ analyses were congruent and differed only in the resolution of the taxa relationships. In the maximum likelihood phylogram of SSU loci (Figure 5.2), the photobiont of *P. stigonemoides* clustered in clade 1 (Jackknife support 100 in all three phylogenetic analysis), which includes *Auxenochlorella protothecoides* (Krüger) Kalina et Punčochářová, the endosymbiont of *Hydra viridis*, an unidentified *Chlorella* species (Huss et al., 1993/94) and *Prototheca wickerhamii* which is an opportunistic pathogen of animals and humans having a weak immune system and is also associated with tree pathology (Pore et al., 1983). Two short inserts (20 and 28 nucleotide long) were found in the sequenced part of SSU in the *P. stigonemoides* photobiont isolate at position number 214 and 942 (compared with X74003.1, *Prototheca wickerhamii*). The photobiont of *P. epiphylla* grouped with clade 2 (Jackknife support 99 and 96 in MP and ML analysis respectively), being closest related with the non-symbiontic alga *Chlorella luteoviridis* Chodat in Conrad & Kufferath and these were sister to *Chlorella saccharophila* and *Pseudochlorella* sp.

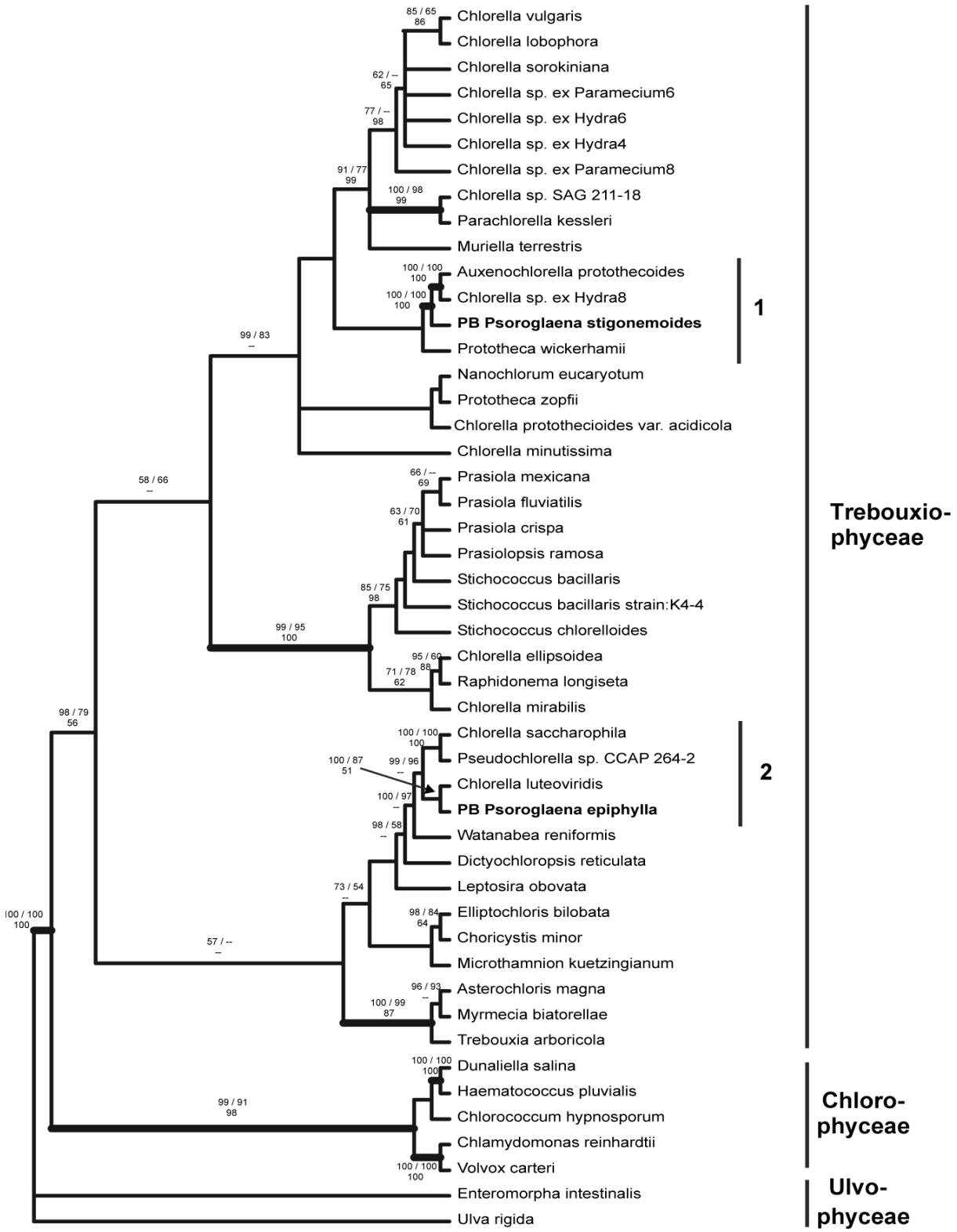

Figure 5.2 Consensus tree of the two most likely trees resulting from the ML analysis of SSU sequences. Jackknife values given were calculated separately for 500 replicates of a weighted MP (first row, first number) and a NJ analysis (second row) and 100 replicates of a ML analysis (first row, second number) and indicated at the corresponding branches. Bold lines indicate >80% Jackknife support in all three methods. PB: photobiont of lichen forming ascomycetes. Sequences obtained from database are indicated in table 1. The tree is outgroup rooted with sequences of ulvophyte taxa.

5.4.3 *rbc*L phylogeny

The large subunit (*rbc*L) of Rubisco (ribulose-1, 5-biphosphate carboxylase/oxygenase) is encoded by the plastid genome itself. *rbc*L sequences have been used extensively for assessing phylogenetic relationships among plants and algae. In the present study the *rbc*L loci of *Psoroglaena* photobionts and several other green algal lichen photobionts and non-symbiotic taxa were analyzed (Table 5.1; Fig. 5.3). The consensus trees obtained by the MP, ML and NJ analyses were congruent and differed only in the resolution of the taxa relationships. As in the SSU analyses the photobiont of *P. stigonemoides* turned out to be most closely related to *Auxenochlorella protothecoides* in clade 1 (Jackknife support 100 % in all three phylogenetic analyses), but the *rbc*L sequence obtained from whole lichen DNA extract of *P. epiphylla* clustered just outside *Prasiola* and not, as expected, with *Chlorella*. The whole procedure of DNA extraction and processing was repeated three times, always leading to the same result. As a comparatively large area of the microfilamentous thallus was investigated with SEM techniques and only very few epibionts observed we conclude that the photobiont made up distinctly more than 95% of algal biomass in the samples investigated. We therefore assume having sequenced photobiont DNA. However, when using DNA extracts from lichen samples collected in the wild one can never fully exclude having amplified contaminating epibionts. One possible interpretation of the result obtained might be different mutation rates of SSU and *rbc*L loci in the photobiont of *P. epiphylla*. A similar situation was described by Hayden & Waaland (2002) in Ulvaceae and by Sherwood et al. (2000) in Prasiolales.

In the present study a very short part of photobiont LSU between positions 642-1113 of the reference sequence Z95381 were sequenced (data not presented). The LSU sequence turned out to be highly conserved across a wide range of algal taxa. Therefore no resolution was obtained in phylogenetic analyses. In the LSU of the *P. stigonemoides* photobiont isolate a 63 base long insert was found at position number 677 (position compared to *Trebouxia arboricola* LSU sequence; Z95381).

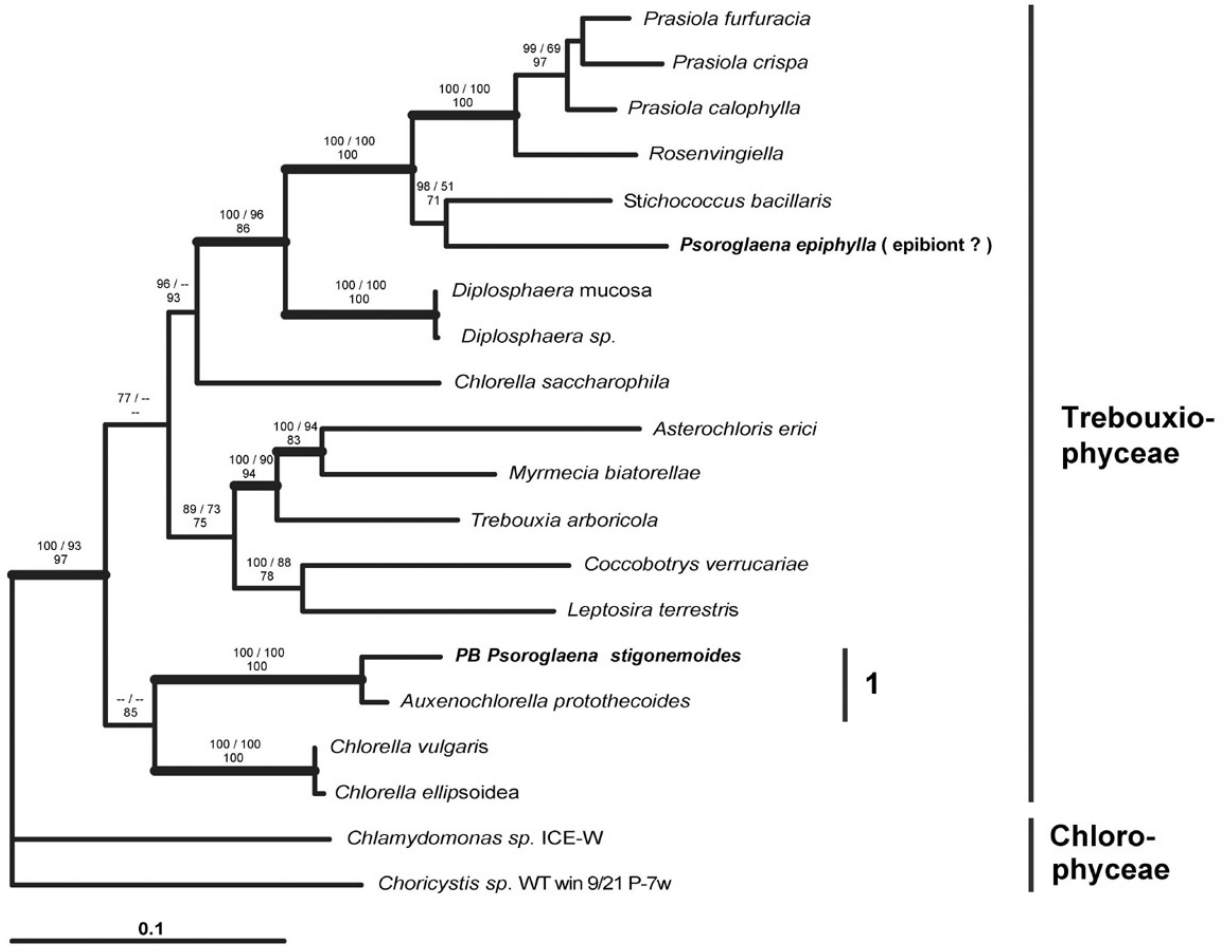

Figure 5.3 Single most likely phylogram resulting from the ML analysis of the rbcL locus. Jackknife values given were calculated separately for 500 replicates of a weighted MP analysis (first row, first number), a ML (first row, second number) and a NJ analysis (second row) and indicated at the corresponding lines. Bold lines indicate >80% Jackknife support in all three methods. PB: photobiont of lichen forming ascomycetes. Sequences obtained from GenBank are indicated in table 5.1. The tree is outgroup rooted with sequences of chlorophyte taxa.

5.5 Discussion

Our ultrastructural and molecular data sets demonstrate that the photobionts of *Psoroglaena epiphylla* and *P. stigonemoides* do not belong to the prokaryotic Prochlorophytes, as suggested by Henssen (1995), but are unicellular green algae of the class Trebouxiophyceae *sensu* Friedl (1995). The photobiont of *P. stigonemoides* is closely related to *Auxenochlorella protothecoides*. This is the first report of this kinship of unicellular green alga as a photobiont of lichen-forming fungi, but interestingly another close relative has been described as *Chlorella* symbiont of Hydra

by Huss et al. (1993/94). Thus some symbiotic “predisposition” might occur in this lineage. The photobiont of the subtropical to tropical *P. epiphylla* is closely related to *Chlorella luteoviridis*. *Chlorella* spp. are known to be the photobionts of protists (ciliates, foraminiferans and other amoebae), invertebrates (Cnidaria), and have also been reported from few lichen-forming ascomycetes in the Caliciaceae and in the genera *Lecidella*, *Micarea* and *Trapelia* among the Lecanorales (Tschermak-Woess, 1988; Friedl and Büdel, 1996; Huss et al., 1999).

According to the presented data the emendation of the genus *Psoroglaena* Müll. Arg., as proposed by Henssen (1995), is inappropriate. The central statement “the genus *Psoroglaena* is restricted … to species having a filamentous cyanobacterium as photobiont” (p. 205) turned out to be wrong. Representatives of the genus *Psoroglaena sensu* Henssen (1995) have more or less distinct, knobby warts on their peripheral hyphal walls (Fig. 5.1b, d-e), a feature known primarily from ascospore walls, but not from vegetative hyphae. They share this feature with representatives of the verrucarialean genera *Leucocarpia* and *Agonimia*, which now also includes the monotypic genus *Flakea* (Eriksson, 1992; Aptroot and Diederich, 1997). The whole complex deserves a thorough phylogenetic analysis.

An even more fascinating result of the present investigation is the fact that the shape of the microfilamentous thalli of *Psoroglaena* spp. is not influenced by a filamentous photobiont, but produced by the fungal partner in symbiosis with a population of unicellular green algal cells. The photobiont cells are brought in uni- or multiseriate arrangements by the fungal partner, which indeed strongly resemble the colonies of filamentous cyanobacteria. This is the first report that microfilamentous thalli can be formed by lichen mycobionts harbouring unicellular photobionts.

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